IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Attorney Docket No. 038007/0111

In re application of

Peter K. Law

Serial No. 09/005,034

Filed: January 9, 1998

Examiner B. Brumback

Group Art Unit 1642

#20
KD
7-13-00

For: MYOBLAST THERAPY FOR COSMETIC TREATMENT

APPELLANT'S BRIEF PURSUANT TO 37 CFR § 1.192

Commissioner for Patents and Trademarks
Washington, DC 20231

Sir:

This brief is in furtherance of the Notice of Appeal filed in this case on March 6, 2000, the period for response having been extended by the accompanying petition for extension of time and fee. The fee required under §1.17(f) and the fee required for payment of an extension of time is included in our attached Check No. 4276 in the amount of $340.00. Any fee deficiency or overpayment may be charged to our Deposit Account 19-0741. This brief is transmitted in triplicate in conformance with 37 CFR §1.192(a).

1. REAL PARTIES IN INTEREST

The captioned application is un-assigned and still owned by Peter K. Law, the inventor and applicant.

07/07/2000 SDUONG 00000034 09005034
01 FC:219 150.00 OP

2. RELATED APPEALS AND INTERFERENCES

There currently are no appeals or interferences involving any related applications.

3. STATUS OF CLAIMS

All pending claims, namely claims 20-25 and 27-32, stand finally rejected, and are the subject of the present appeal. Claims 1-19 and 26 were canceled without prejudice or disclaimer in the amendments filed January 9, 1998 and June 9, 1999 respectively.

4. STATUS OF AMENDMENTS

A final rejection was mailed on October 6, 1999, which entered Appellant's claim amendments filed on June 9, 1999. After receipt of the final rejection, Appellant filed on February 7, 2000 an amendment and request for reconsideration under 37 CFR § 1.116. In an advisory action dated March 30, 2000, the examiner refused to enter the response after final rejection. The attached claim appendix sets forth the claims in the form prior to submission of the response after final rejection.

5. SUMMARY OF THE INVENTION

The claimed invention is a method for transferring myogenic cells into a tissue to build up the mass of that tissue. Myoblasts are the only known cells in the body capable of natural cell fusion, and the inventor has discovered methods for incorporating larger amounts than usual of exogenous cultured myoblasts (page 5 top, specification) into tissues that contain pre-existing muscle and fat that the myoblasts attach and fuse to. The inventor has discovered the importance of: (1) maximizing cell fusion by injecting greater (more than 10 times as much compared to the prior art) numbers of cells for each injection, due to set minimum losses from the procedure itself, (see page 25 lines 9-10); (2) injecting transversely to minimize those losses, (see Fig. 6 and page 16 lines 16-28 of the specification); (3) using highly purified cells lacking fibroblasts (see p. 20 lines 20 - 21) (4) optional use of added chondroitin sulfate in the injected material to induce cells to fuse and minimize the losses further, (see page 19 line 30 to page 20 line 31); and (5) use of

"superior cell lines of myoblasts (that should be highly) nonantigenic" see page 23 lines 4 to 9 and the related description on pages 23 and 24. The inventor studied *inter alia*, these five factors from his insight that each was particularly important for succeeding in this field where no one had succeeded before.

Appellant found the listed factors to be particularly important, and that they were not sufficiently appreciated prior to applicant's discovery, resulting in the failure of others in the art who tried to apply similar methods. The inventor has emphasized this role for his discovered factors as for example explained on page 26, lines 8-10, "the technique of myoblast delivery is essential for MTT (myoblast transfer therapy) success."

The discovered technique particularly relates to and makes possible the implantation of a greater number of cells, which is highly important to the claimed methods of cosmetic alteration. The inventor was astonished to find that that limbs of patients that had received his high cell number implantation therapy treatment not only showed altered morphology and evidence of dystrophin production by the implanted cells, but that they were noticeably larger (see exhibit A that was previously provided as Appendix D in a February 7, 2000 amendment and response). That is, the methods additionally could "bulk up" selected tissues and thereby alter the appearance of a patient. The claimed invention concerns treating body parts that contain muscle and fat cells such as breast (breast muscle and fat), hip (hip muscle and fat) and face (facial muscles and fat) as a cosmetic treatment therapy (see page 12, lines 11-12).

6. ISSUES

A. Whether the Examiner erred in rejecting 20 - 25 and 27 on enablement grounds because, inter alia "detailed teachings are necessary to overcome the conventional wisdom" as argued on pages 3-6 of the first Office Action, and maintained in item 5 (p. 2-6) of the Final Office Action.

B. Whether the Examiner erred in rejecting claims 28 - 32 on enablement grounds because inter alia, "detailed teachings are necessary to overcome the conventional

"wisdom" as asserted against claims 20 – 27 but also for additional reasons as asserted on p. 3 – 6 of the Final Office Action.

C. Whether the Examiner erred in rejecting claims 31 and 32 on enablement grounds because the prior "art teaches that surgical implants are performed without dissecting the body part from the individual."

D. Whether the Examiner satisfied her burden of showing that claims 31 and 32 are indefinite for omitting a step of "re-insertion of the body part after dissection."

E. Whether the Examiner erred in relying on a 7 year old personal attack that itself was subject of a defamation lawsuit between Appellant and the cited author, to support non-enablement on the basis that "the art teaches that human transfer has not been successful or at best that the results of human transfer have been controversial."

7. GROUPING OF CLAIMS

For the purposes of this appeal only, the claims can be placed into the following groups:

Group I – claims 20 – 25, 27;

Group II – claims 28 - 30;

Group III – claims 31, 32.

Groups II and III do not stand or fall together with Group I because only Group I recites "culturing myogenic cells to form a composition of cells," and arguments presented in the first Office Action are directed to this language. Group II does not stand or fall together with Group I or with Group III because only Group II recites a method for "replacing injection of silicone with multiple transverse injection," and arguments presented in the first Office Action are directed to this language. Group III does not stand or fall together with Group I or with Group II because only Group III recites a method for "dissecting and removing tissue from the body part" and "surgically implanting myotubes

into the body part." These groupings will be further understood in view of the discussion below.

8. ARGUMENT

All claims are rejected on enablement grounds under 112 first paragraph. The Federal Circuit in *In re Wands,* 858 F.2d 731, 8 USPQ2d 1400 (Sep. 30, 1988) provides 8 well-known "Forman Factors" for use in resolving enablement issues, as dictated in MPEP 2164.01. Appellant argues each factor in the order suggested by *In re Wands* for resolving whether "undue" experimentation is required to practice the invention:

1. The quantity of experimentation necessary.

2. The amount of direction or guidance presented.

3. The presence or absence of working examples.

4. The nature of the invention.

5. The state of the prior art.

6. The relative skill of those in that art.

7. The predictability or unpredictability of the art.

8. The breadth of the claims.

Forman Factor 1: **Little, if any experimentation is necessary to practice the invention**

The invention as described in the specification does not require extensive optimization or trial to carry out successfully. What optimization therein implied, exists as a routine procedure of medical practitioners, who are very highly skilled and who adapt their highly specific methods and medicaments to each patient on an individual basis. In fact, the highly skilled medical practitioners routinely study the three dimensional layout of a patient's skeleton and musculature, using X-rays, sonograms and the like. Unlike the experiments reported in the specification, such review is routine. That is, when conducted on a mouse we call the work an experiment, but such review of muscle groupings for each animal is easier to carry out in a human patient by a highly skilled practitioner who has spend years studying and manipulating muscles of that species, contrary to the Examiner's argument at the bottom of page 3, from the first Office Action.

The real examples covering two species provided in the specification do not utilize new experimentation, but teach numbers of cells, types of injections and the like. For example, the specification points out that "oblique injection has been used" as a preferred method for "clinical trials" on humans but that "the myofiber orientation of different muscle groups have to be well-studied by the orthopedic surgeons who administer myoblast injections." This does not suggest that experimentation is required for regular practice of the invention. It merely teaches the orthopedic expert who has spent years training to observe and manipulate myofibers, that the best mode to Appellant was "oblique injection" and suggests where further improvements could be made in the future. The Examiner has refused to acknowledge this point, arguing instead that this passage points "more toward increased unpredictability in humans" (page 3 middle, page 4 top line). It is wrong to attack a suggestion for further improvement to an example in a disclosure as evidence of "increased unpredictability," and this argument against enablement is not correct on its face.

Appellant has provided very detailed guidance, eliminating the need for undue experimentation. For example, the specification teaches the exact numbers of cells which have been successfully transferred into specified human tissues (page 12 lines 8 to 12), the

number of injections (lines 7-8) which are needed but have an upper limit, the types of injections (Figure 6, page 25 and best mode mentioned on page 26 line 11), and purity with respect of fibroblasts (page 20 line 21) and the types of cells (page 23 line 4 to page 24 line 34). Appellant has provided a chemical concentration range of optional chondroitin sulfate to use and has cautioned against using cells that have undergone excess cell divisions (pages 20 lines 29 – 30 and page 31 lines 22 - 30 respectively respectively). For example page 20 lines 29 to 31 teaches using chondroitin sulfate "from approximately 5 uM to about 5 mM in the transfer medium." This range, which is recited in claim 29, is not overly broad in consideration that all drugs and ingredients have a wide range of activity and are optimized for final medical use as determined by the Food and Drug Administration, not the Patent and Trademark Office. Appellant has carried out the described methods many times and the methods described work well, if followed correctly. Appellant even has studied why others did not succeed before him and has reviewed factors that others before him have ignored. These factors are present in the specification and reviewed here but also reviewed in the literature (Exhibit E, particularly p. 96 right column middle to bottom and p. 98 left column middle to bottom).

Since his work, others have followed in Appellant's footsteps but have not reported the need for particular experimentation to achieve his success. That is, Appellant is not aware of any limitation of his methods that were overcome by a new discovery or optimization required to achieve success and the Examiner has not provided any such evidence. In fact, the principal reason for lack of success by others has been their reluctance to invest the time and money in preparing suitable numbers of cells and to inject them obliquely to obtain a sufficient amount to make a difference to the recipient, both in a biochemical sense and a cosmetic sense. This is the main reason for the failures discussed by Hoffman and Coovert, but the Examiner has refused to admit this point. The work cited and summarized in the citations (Hoffman and Coovert, middle of page 4, first Office Action) did not follow Appellant's methods and certainly did not use nearly enough cells. More importantly, the Hoffman article contained a personal attack on the moral integrity of Appellant, inpugning his reputation in two places and criticizing his leaving academia to start a business. Accordingly, rather than providing evidence that undue experimentation would have been required to practice the claimed invention, Hoffman uses a distinct,

flawed methodology and merely asserts a personal vendetta against Appellant that resulted in a public apology, a copy of which has been entered into the record (February 7, 2000 amendment) and is reproduced as Exhibit C.

Forman Factor 2: **The Specification provides Specific Guidance that Overcomes prior art Failure**

The specification teaches certain "enabling factors" that overcame prior art failure. Since the 1992 publication date of the Hoffman reference that the Examiner uses as evidence of non-enablement, Appellant's method described in the specification has become accepted. Evidence of that acceptance (designation of Fast Track development program status for Appellant's Investigational New Drug Application) was provided in Appellant's response filed February 9, 1999 a copy of which is attached as Exhibit F. Appellate has studied the failings of others, found how to succeed based on certain new methods and has incorporated the new methods needed for success into his patent specification.

The same enabling features in the specification were outlined in a scientific paper published 5 years after the Hoffman reference and referred to earlier in prosecution (Exhibit B). See Gene Ther. Mol. Biol. pp. 345-363 (March 1998), which specifically "reconciles (Appellant's) positive results with less convincing ones" published between 1992 and 1995 (see page 350 column 2 second to the last paragraph). In other words, as evidenced by this reference, over the years, Appellant has carefully examined why his system works and has identified the factors that are needed to enable practice of successfully transplanting large enough amounts of material to cause a cosmetic change. These "enabling factors," which particularly relate to cosmetic alteration are listed both in the scientific paper and in the present patent application.

Enabling Factor 1: "To begin with, the use of large quantities of pure live myoblasts is a pre-requisite" and "Myoblast cultures are usually contaminated with fibroblast overgrowth."

Appellant's research paper describes "To begin with, the use of large quantities of pure live myoblasts is a pre-requisite of successful MTT. Except for one study (by Appellant), there is no published pictorial evidence to substantiate the purity, myogenicity and viability of the injected myoblasts as claimed. Myoblast cultures are usually contaminated with fibroblast overgrowth." (page 350 last two paragraphs). Appellant has described this enabling factor in the specification see page 20, lines 20-21: "It is therefore, necessary to inject as pure as possible fractions of myoblasts in MTT (myogenic cell transfer therapy) without contaminating fibroblasts" along with a biochemical explanation for why contamination with even small quantities of fibroblasts in the implanted compositions prevent success (lines 10 - 20). The 1998 research publication of Appellant cited by Appellant (Exhibit B) mirrors this enabling explanation with the instruction that "MTT with such impure culture could lead to deposition of connective tissues rather than myofiber production" (last full sentence on page 350). Thus, Appellant has not only identified and described this enabling factor but he subsequently has discovered a believed biological basis for why this new factor is necessary to enable the invention, and which was under-appreciated by previous workers.

Enabling Factor 2: Culturing tens of Billions of "Pure Human Myoblasts" from muscle biopsy

Prior art reports of myogenic cell transfer were for "work at ranges of hundreds of millions of myoblasts" or about 100 times less (sentence bridging pages 350 - 351 of Appellant's 1998 research paper) that the technique taught in the specification. The specification teaches that, in contrast to that used in failed attempts of others a higher range for example on page 12 top "12.5 billion myoblasts, to either 28 muscles in the upper body (UBT) or to 36 muscles in the lower body (LBT)" are needed. Appellant discovered, in using such large amounts, that it is very important that those compositions be free of fibroblasts, as taught on page 20 lines 20-21 of the specification. This enabling factor is particularly important for cosmetic purposes where the mass transferred is important.

Enabling Factor 3: Too many injections for cell delivery causes injection trauma

Applicant has found that a large number of cells must be administered because regardless of the total number of cells transferred, a given (fairly fixed) amount is destroyed, necessitating a much greater amount to add per location and per muscle. Appellant's research paper explained the reason that (page 351 second paragraph) "studies that reported failure .... Subscribed to the fallacy of making 55 to 330 injections into a muscle the size of an egg, traumatizing indiscriminately the underlying nerves, muscle, and vasculature. These injection traumas boosted macrophage access and host immune responses...They also induced fibrosis..." The specification teaches, and in fact strongly emphasizes this important enabling feature that allowed Appellant to succeed where previous workers failed. See for example page 25 lines 9-10 "The goal was to achieve maximum cell fusion with the least number of injections" and page 26 lines 8-10 "the technique of myoblast delivery is essential for MTT success" while noting that multiple injections are unavoidable. Appellant has studied the factor of injection type and teaches the best mode from data described in Figure 6, concluding that "Oblique myoblast injection delivers donor myoblasts to the greatest number and area of recipient muscle fibers with the least leakage, resulting in the formation of the most mosaic fibers" on the middle of page 16 of the specification.

Enabling factor 4: Large Numbers of Cells are Needed to Overcome a Standard Loss

Appellant has explained in his research paper (Exhibit B) that "An allowance in the number of injected myoblasts has to be made to satisfy the unavoidable scavenger process" because "once injected, the myoblasts are subjected to scavenger hunt by macrophages for up to three weeks" (page 351 left side bottom paragraph). This also points out that "The less successful MTT (Myogenic cell Transfer Therapy) teams focused on immunosuppression to prevent T-lymphocyte proliferation and antibody production without overcoming the primary hurdle of

providing enough pure and live myoblasts." The specification emphasizes the need for massive amounts and recites "billions" of cells particularly on page 12. This figure is important when compared with prior art figures in "ranges of hundreds of millions of myoblasts" as summarized at the top of page 351 of the cited paper.

Appellant discovered these enabling factors via experimentation, and the most relevant experimentation is detailed in the specification. The Examiner, however, has dismissed as "irrelevant" most of these results and their considerable guidance on how to practice the invention as provided in the specification, when Appellant pointed them out in response to the enablement rejection. The Examiner provides no explanation why these crucial features, which lay at the heart of the invention are, in the Examiner's words, "irrelevant." See for example page 4 lines 3 to 6 from the bottom.

In fact the most important features of the new methods for cosmetic therapy that enable success over the prior art failures originated with four sets of studies that are reported in the specification. The first study was on humans and demonstrated that larger cell numbers than had been previously appreciated could be used successfully for long term establishment of new tissue. Boys that had muscular dystrophy each received 12.5 billion myoblasts via 200 injections and showed muscle improvement that was determined both morphologically and immunochemically (page 12 lines 4-31, page 15 lines 3-6, and Fig. 19). Prior art studies, including those cited by the Examiner and also summarized by Appellant in his 1998 paper only used 10 fold or fewer cells. A copy of that paper is provided in Exhibit E (see page 96 column 2, 16 to 19 lines from the bottom). This difference is not irrelevant.

The second study investigated myoblast distribution and survival (cell fusion rates, glucose phosphate isomerase activity and morphology) as a function of the angle of injection, using mice. See the specification page 25 line 7 to page 26 line 18 and Fig. 6. The results from that study (see Figures 7 and 8, and page 25 line 27 through page 26 line 10) indicated that "the technique of myoblast delivery is essential for MTT (myoblast transfer therapy) success" and that the most concentrated suspension possible should be

delivered. Further, the actual method used to obtain successful clinical results was the "oblique injection" (see page 26, line 11).

A third study, the details of which are not presented in the specification, was performed on mice and indicated that chondroitin sulfate should be added to the transfer medium. More specifically this material should be present "(ranging from approximately 5uM to about 5mM) in the transfer medium" (see page 20 lines 22-31). This information is recited in claims 27 and 29.

A fourth factor that was important to enable the invention was use of MHC-I antigen deficient cells. A fourth study demonstrated purification of MHC-I antigen deficient cells from cultured human myoblasts. See page 23 line 4 to page 24 line 34 and Figs. 2, 3, 4, and 5.

Working under extreme limitations that prevent most studies with human beings as medical subjects, Appellant has carried out his first work on multiple dystrophy patients, who had very poor prognosis and for which the studies were justified on humanitarian grounds, and not merely experimentation. It is normal for initial human studies to take place with medically compromised patients, such as the case here. The Examiner has alleged that the invention will only work with normal animals, and Appellant has provided data from normal mice studies. In the absence of work done on normal humans, the PTO accepts data from a model system unless there is a scientific reason for rejecting such data. In fact, Appellant carried out numerous basic research studies on a mouse system and the disclosure result from those studies as outlined above.

FormanFactor 3: **Two Working Examples are Provided in the Specification**

The Examiner has stated on page 4 line 10 of the first Office Action that "the disclosure only teaches myoblast transfer into mice." Thus, the examiner admits that at least one working example with mice was present in the specification. In this context Appellant points out that independent claim 20 and dependent claims thereon recite "subject" and other claims recite "body part" while the specification mentions that the

invention pertains to "mammals" (for example line 12 of page 1). The plain meaning of the claims, unaltered by limitations read into them from the specification, include not only human but also other mammals such as the mouse. Accordingly, as the Examiner pointed out, at least one acceptable working example is provided in the specification.

In addition to the working example admitted to by the Examiner, Appellant, as limited by ethical restrictions as described above, provided a second working example of treating dystrophic boys (page 12, lines 1 to 31). Appellant has provided picture evidence of cosmetic effects from that treatment method and provide another copy of that picture as Exhibit A attached herein.

The Examiner has argued that the second working example is not real because "the art teaches (myoblast cell) incorporation occurring in regenerating muscle to a much greater extent than in normal uninjured or non-diseased muscle" and "it is unclear how donor myoblasts could be used to alter the appearance of non-diseased muscle" (first Office Action page 5 lines 9 to 14). As cited above, appellant has working examples in both normal (mice) and diseased (human) animals. These working examples directly contradict the Examiner's reasoning that it can only work for one or the other. The examiner thus has failed her duty to provide evidence that undue experimentation would have been required, and the evidence of record flatly contradicts the reason for the enablement rejection.

In other words, the argument fails for two reasons. One reason is that claim 20 refers to a method for "treating a body part of a subject" and is not limited to only "normal uninjured" muscle. Thus, enabled matter within the scope of this claim exists by way of working example. Appellant submits in this context that "the examiner should always look for enabled, allowable subject matter and communicate to applicant what that subject matter is at the earliest point possible in the prosecution of the application" (MPEP 2164.04) but has not done so.

The second reason is that NO tissue implanted according to the invention is "normal uninjured or non-diseased muscle" because all tissue according to the methods described and claimed must be injured by piercing. In fact, the minimum injury created by the

method itself is so great that Appellant has emphasized the need to limit it "The goal was to achieve maximum cell fusion with the least number of injections" (see page 26 lines 8 – 10). Furthermore, Appellant has studied the effects of this injury and has measured a number of biochemical changes indicating that this step of the procedure causes traditional injury responses such as "boosted macrophage access and host immune responses" and induction of "fibrosis." Without wishing to be bound by any one theory of this embodiment of the invention, Appellant believes that the important variable here is that some injury (62.5 million cells administered per needle stick for about 5 to 7 injections per muscle as described on page 12 lines 8 - 10 of the specification) facilitates fusion to disaggregated fibers but that too much injury caused by prior art procedures ("from 55 to 330 injections into a muscle the size of an egg" as described on page 351 second paragraph of Appellant's 1998 research paper submitted in a response (see Exhibit B) is very deleterious.

The body parts of dystrophic humans (second working example) were altered. Appellant do not claim a particular degree of alteration of cosmetic appearance and previously have submitted a picture showing a cosmetic appearance change in subjects treated according to the methodologies presented herein.

The Examiner alleges that "it is unclear how donor myoblasts could be used to alter the appearance of non-diseased muscle" (first Office Action page 5 lines 6-7 from bottom). This criticism appears directed to the part of the claim scope that covers treatment of non-diseased body parts. However, Appellant has provided evidence of use in non-diseased muscle of one species (mouse) included in the scope of the claims. The Examiner has not acknowledged the existence of this data in this context. Furthermore, as Appellant has pointed out previously, multiple injections, as described in the specification produce (unavoidable) injury and such injury occurs even for non-diseased tissue during practice of the invention. Without wishing to be bound by any one theory of this aspect of the invention, Appellant believes that ripping muscle cells with a needle stimulates their re-fusion and regrowth at a time when myoblasts are transferred. Of course, there is a limit to this and this limit is one reason why each injection has so many more cells than that previously used by others. This also seems to be the reason why moderate exercise (which

facilitates the success of the claimed method) works (and "strenuous exercise causes damage") and is a preferred embodiment (see page 27 lines 5 to 7 of the specification). Applicants also point out that even if less re-fusion occurred in healthy tissue, the method still works, because fusion is not blocked.

Forman Factor 4: **The Nature of the Invention**

The invention concerns the manipulation of cells by culturing and then administering cells to a mammal, preferably a person. This invention is not to a specific chemical or physical composition but to living material that is manipulated and placed into a unique living organism and is to be carried out by a medical arts practitioner. The factors that Appellant found to be crucial for success over the previous art concern not only the numbers of cells but also the mechanism of their transfer. In fact, most if not all of earlier failed attempts did not use nearly enough cells due to this nature of the invention. This, and the other desirable inventive manipulations are critical for success.

Forman Factor 5: **The state of the prior art was sufficiently advanced to enable the invention when combined with Appellant's teachings**

The Examiner has admitted that the art of "human transfer" may "have been controversial" on the priority date (page 4, lines 5-6 of the first Office Action). In other words, although there was some agreement that "human transfer" had not been done, the art was so close that this issue itself was controversial. That is, some believed that "human transfer" was a reality, even without Appellant's disclosure. Accordingly, the level of experimentation and of guidance needed was not so high that consensus opinion held that success was not at hand or was impossible. Appellant's work, at the priority date, in fact, occurred in an environment wherein many coworkers already felt that human transfer was possible. Moreover, Appellant soon after published his successful results obtained using the methods described in the specification.

Forman Factor 6: **The Relative Skill in the Art Was High**

The level of skill in the art was very high, and the skilled artisan certainly was familiar enough with basic manipulation and transplantation of cells. The claimed invention is directed towards a highly select group that itself is selected from highly skilled medical doctors who already have years of training in internal surgery and/or cosmetic surgery. Furthermore, the invention is being readied to be carried out in medical centers among teams of experts in centralized locations and is not to be practiced by an average physician. It is customary for a practitioner in this environment to spend many hours training before utilizing a new medical procedure. Accordingly, the relative skill of users of the invention is unusually high when compared with that of skilled artisans in other fields. Such highly gifted, trained and motivated experts at the time of filing the priority document did not need that much direction in order to carry out the invention as described in the specification.

Forman Factor 7. **Appellant's Method Solved the Prior Art Predictability Problem**

On page 4 lines 8 and 14 of the first Office Action the Examiner asserts that "due to the unpredictability of the art, detailed teachings of the claimed method are required." The explanation given for "unpredictability" was that "the art teaches that human transfer has not been successful or at best that the results of human transfer have been controversial" (lines 5-6). Applicants do not challenge the Examiner's conclusion that the two references cited (Coovert and Hoffman) cite work that failed to obtain successful cell transfer. However, as mentioned on page 5 of their June 9, 1999 response, Appellant had discovered that a certain amount of cells are lost during transfer and that they had to greatly increase, by more than 10 fold the number of cells transferred per injection and had to carefully eliminate fibroblasts from injected material. These important differences are a matter of record, having been discussed in a submitted document, a copy of which is attached as Exhibit E (see page 96, right column lower half).

It is very significant that the referenced studies failed to appreciate the need for vastly superior quantities. Moreover, this particular unappreciated element of the invention is directly responsible for Appellant's ability to obtain cosmetic changes where others had

not before. Appellant believes that previous workers expected that administered cells would only bind with preexisting muscle cells and that adding more cells would not necessarily be as effective because there were too. On the other hand, unlike those other cited workers, Appellant exerted much more effort to prepare larger numbers of cells and found that the opposite was true. That is, if only some are implanted all seem to be lost but if many excess are attempted, a greater proportion seem to take hold.

Without wishing to be bound by any one theory of his invention, the inventor believes that adipocytes reacted with the excess cells to allow a greater mass to build up, as he explained in Appendix D submitted previously to the Examiner on February 7, 2000, "Apparently myoblasts can survive and develop when injected into adipose tissue including the breast, hip, check (sp.) buttock, etc. (Satoh et al, 1992)." A copy of that referenced Satoh article is provided as Exhibit D. The view that myogenic cells can fuse with fat cells thus is supported by others. For example, (as previously submitted in Appendix D) Teboul indicates that myogenic cells such as myoblasts can be converted into fat cells to augment the size, shape and consistency of soft-tissues (Teboul et al. 1995). Thus, the techniques taught are applicable to the use of myogenic cells to augment body parts that contain fat cells. The fact that this condition had not been studied previously does not mean that the prior art was not predictable, only that the prior art did not employ the right conditions for success.

Forman Factor 8: **The Claims are Not Overly Broad with Respect to the Examples**

The Examiner has argued that "the disclosure only teaches myoblast transfer into mice" but that the claims "encompass treatment of humans" (first Office Action page 4 lines 10 and 2 respectively). The Examiner also alleges that, for claims 28 – 32, the specification "describe[s] methods for myoblast injection 'into the gastronemius muscles of twenty normal 3-month old normal mice' not humans," implying that studies in mice do not relate to another mammal within the group contemplated. However, the Examiner cites no evidence that work done on mouse model systems does not relate to humans. Furthermore, the Examiner has not presented any adequate reason to support the allegation of species-to-species differences. Accordingly, the claim scope is not necessarily broad. As stated in

MPEP 2164.02 "Proof of enablement will be required for other members of the claimed genus only where adequate reasons are advanced by the examiner to establish that a person skilled in the art could not use the genus as a whole without undue experimentation."

In this context, Appellant points out that the specification provides proof that the invention is enabled in humans (page 11 bottom through page 12). A picture showing cosmetic alteration in dystrophic humans was provided, and a copy of that same picture is attached as Exhibit A. The Examiner has dismissed the significance of the examples using diseased humans due to claim breadth, arguing that "Applicants claims are not limited to those conditions, but encompass cosmetic alteration of non-diseased body parts" (page 4 7 - 8 lines from the bottom). This enablement argument based on claim scope is improper because no "adequate reasons" were advanced by the Examiner, who simply dismissed the example of diseased human as being too different from non-diseased humans. Applicants point out that such comparisons often are made and accepted in the medical community, at the FDA and at the patent office. The Examiner has failed to meet her burden of providing a scientific basis for dismissing this particular application of the invention in diseased humans to non-diseased humans. Appellant further points out that many other more common embodiments include diseased humans, such as mastectomy patients, and these were not considered by the Examiner.

Breadth of Claim 21: The Examiner argues that claim 21 is overly broad (page 4 first Office Action) because this claim recites "face, breast, hip, or non diseased muscle" and "conventional wisdom teaches that myoblasts only fuse with myoblasts (i.e. muscle cells)..... it is unclear how administration of myoblasts to breast tissue (which is composed of adipose tissue) or to a hip (which is composed of bone) could be used to alter the cosmetic appearance of the breast or hip." This argument is wrong for two reasons. One, breast includes muscle tissue (in the context of the invention the underlying breast muscle is used as a scaffold) and hip includes muscle, both facts which are well known to medical practitioners. A second reason is that myoblasts do fuse with adipocytes (fat cells) as described in previously submitted Appendix D which refers to work from two different researchers that agree on this topic: "Apparently myoblasts can survive and develop when

injected into adipose tissue including the breast, hip, check (sp.) buttock, etc. (Satoh et al, 1992) and (Teboul et al. 1995)." (bottom of first page).

The Examiner also argues that the art "does not teach any resulting alteration in the 'cosmetic appearance' of the muscle" (page 4, 9 lines from bottom). Appellant agrees that the art of 1993 did not teach alteration of the cosmetic appearance of muscle and points out that the present claimed invention is directed, *inter alia*, to that effect. The enabling factors discovered by Appellant (reviewed above) specifically are directed to improvements over the art that allow enough successful transfer to allow visible cosmetic changes (see figures of human muscles treated by the claimed invention supplied during prosecution, a copy of which is provided as Exhibit A.)

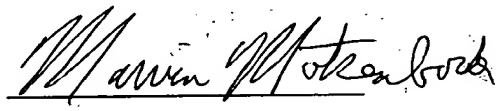
9. CONCLUDING REMARKS

In both Office Actions, the Examiner has opined that "teachings found in the art .... that human transfer has not been successful and is at best controversial." This statement about enablement may have been true in 1993 before the art was informed about Appellant's procedure but certainly is not true today. It is not right that the Examiner primarily cite a 7 year old attack paper against Appellant that was subject of a defamation lawsuit as evidence of non-enablement, particularly in view of the Appellant's teachings both in the specification and in the more recent literature (Exhibits B and E). That is, the 7 year old record evidence and the other old paper cited by the Examiner in the case are not appropriate. Further, the Examiner has not acknowledged the points made both in Appellant's specification and publication based on the same data in the specification, that provides reasons why old attempts summarized in those old papers have failed. The PTO respectfully should accept Appellant's data and discoveries that led to the pioneering invention or come up with more recent and more persuasive evidence why Appellant's data and enabling factors enumerated above are somehow wrong and misguided. Appellant points out that outside of Appellant's own work, no new advances have been presented in the intervening years that anyone has pointed to as evidence of success, despite the fact that the method is accepted and that a number of companies have started to exploit the underlying technology.

10. REQUEST

Appellant submits that the claims are in condition for allowance, and respectfully request reversal of all rejections and allowance of the claims.

Respectfully submitted,



July 6, 2000
Date

Marvin Motsenbocker
Reg. No. 36,614

FOLEY & LARDNER
3000 K Street, NW, Suite 500
Washington, DC 20007-8696
(202) 672-5300
(202) 672-5399

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

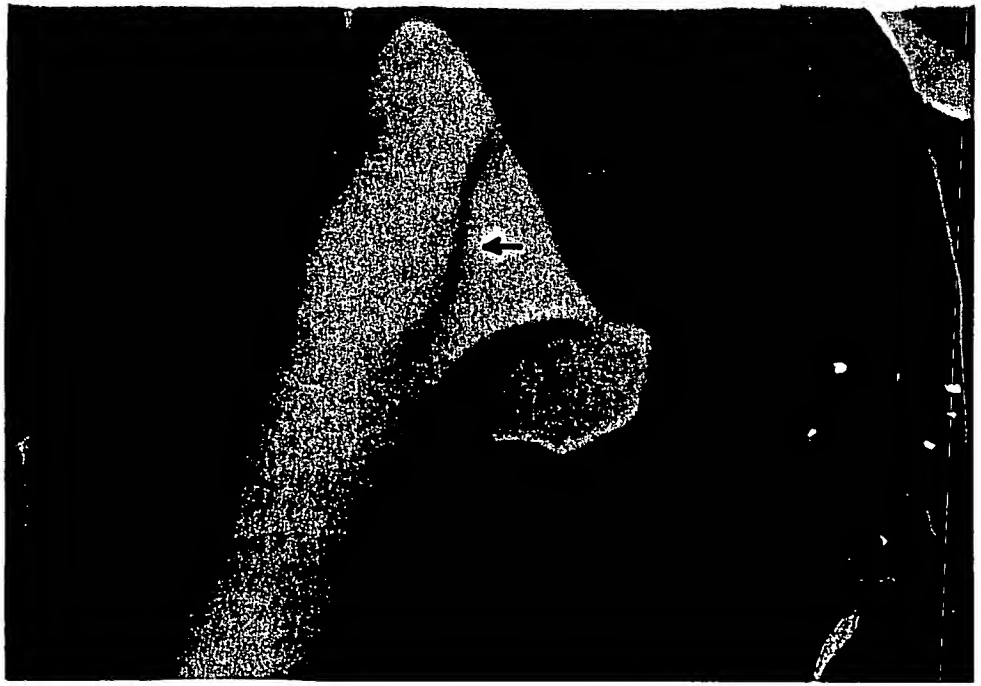
Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

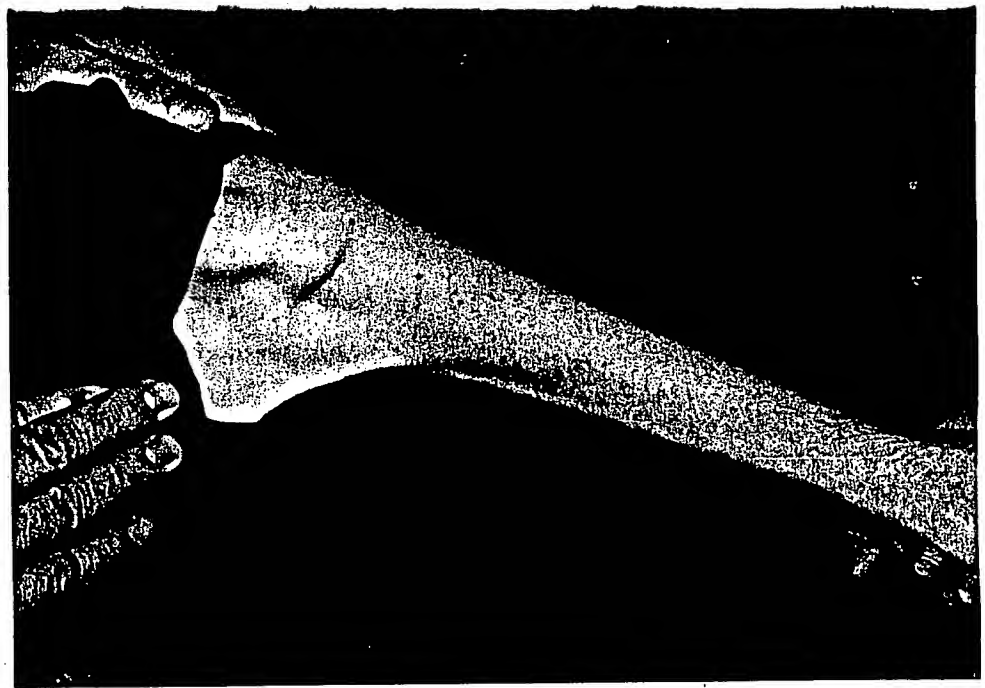
IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images,
Please do not report the images to the
Image Problem Mailbox.

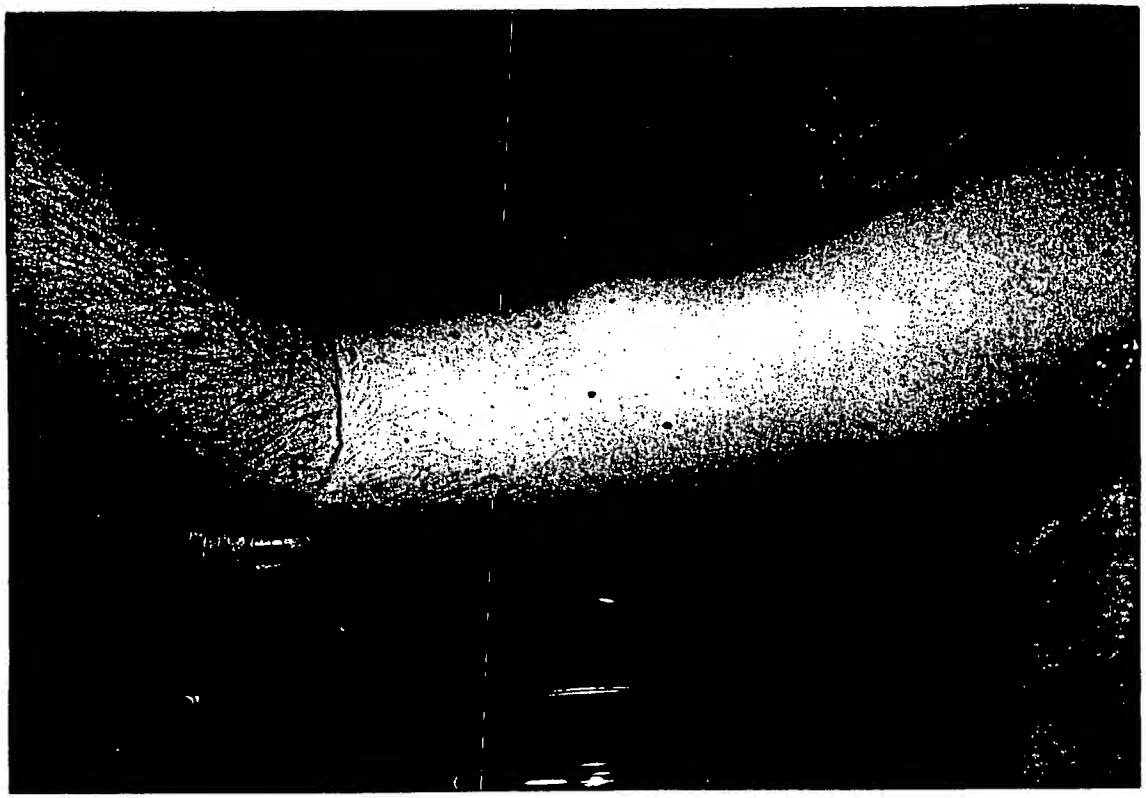
EXHIBIT A



A



B



C

Fig. 1

EXHIBIT B

Gene Ther Mol Biol Vol 1, 345-363. March, 1998.

Myoblast transfer as a platform technology of gene therapy

Peter Law, Tena Goodwin, Qiuwen Fang, George Vastagh, Terry Jordan, Tunja Jackson, Susan Kenny, Vijaya Duggirala, Charles Larkin, Nancy Chase, William Phillips, Glenn Williams, Michael Neel, Tim Krahn, and Randall Holcomb

Cell Therapy Research Foundation, 1770 Moriah Woods Blvd., Suite 16-18, Memphis, TN, 38117, USA

Correspondence: Peter Law, Tel: (901) 681-9045, Fax: (901) 681-9048, E-mail: cell@attmail.com

Keywords: Myoblast transfer; Clinical trials; Gene therapy; Duchenne muscular dystrophy; Viral vectors

Summary

Myoblasts divide profusely, and fuse during muscle regeneration, interiorizing MHC-I antigens and inserting myonuclei with the normal genome into muscles of genetically deficient recipients, where any replacement gene can be stably integrated and naturally expressed. Myoblasts are the natural source and vehicle for many gene therapies. Myoblast transfer therapy is completing US FDA Phase II clinical trials for Duchenne muscular dystrophy.

I. Introduction

The National Institute of Standards and Technology has recently announced that tissue engineering will likely be the key to treating genetic diseases and degenerative disorders that accounted for 50% of the $1+ trillion U.S. health care cost in 1995 (Schwartz, 1997; Langer and Vacanti, 1993; Nerem and Sambanis, 1995).

Among the many programs of tissue engineering, gene therapy has been hailed as the medicine of the 21st century. Despite the nearly universal belief that gene therapy will ultimately allow the treatment of currently incurable diseases and conditions, its potential remains largely unfulfilled (Hillman et al., 1996). Only when a safe and effective gene delivery technology has been proven in humans can the full potential of gene therapy be realized.

To date, over 3000 subjects worldwide have received gene therapies among the 280+ protocols approved. Data indicate that no single vector will serve all systems. In examining gene transfer methods mediated by particle bombardment (Jiao et al., 1993; Sautter et al., 1991), liposomes (Stewart et al., 1992; Ray and Gage, 1992), calcium phosphate precipitation (Ray and Gage, 1992; Albert and Tremblay, 1992), and electroporation (Ray and Gage, 1992; Albert and Tremblay, 1992; Puchalski and Fahl, 1992), one can conclude that transduction efficiency is extremely low and variable. The level of transgene expression depends on the promoter strength in a particular cell type. Only liposomes, together with retroviruses, adenoviruses, adeno-associated viruses and myoblasts have been used in clinical trials.

A. Vectors

1. Liposomes

Cationic liposome/DNA complexes gain cellular entry via receptor-mediated endocytosis (Stewart et al., 1992; Trubetskoy et al., 1992). Assuming the transgene escapes digestion by the endosome, it has no built-in mechanism to get across the nuclear membrane and is therefore non-integrative. The minimal and transient expression of the transgene is the result of random targeting, integration, and regulation. Liposomes have the advantage of being non toxic and can therefore be used in large quantities and repeatedly (Brenner, 1995).

2. Viruses

The viral vectors were the first to gain widespread scientific applications. Notable was "the first federally approved gene therapy protocol, for correction of adenosine deaminase (ADA) deficiency, began on 14 September 1990"(Anderson, 1990, 1992, 1995).

Retroviral vectors can transduce dividing cells with integration into host DNA. They integrate randomly and may cause mutation and cell death. They exhibit no toxicity. Although they can house larger transgenes than adenoviruses and adeno-associated viruses, the capacity is less than 10 kb. They are unstable in primate complement and cannot be targeted to specific cell types *in vivo* (Brenner, 1995; Cornetta et al., 1991).

Adeno-associated viruses and adenoviruses have shown considerable promise and are widely used. They can accommodate a broad range of genetically modified genes; are efficiently taken up by non-dividing cells *in vivo*; do not integrate into chromosomal DNA, thus reducing the risk of insertional mutagenesis; and are amenable to redirected tissue targeting (Morsey and Caskey, 1997).

All viruses can cause harm when they revert to wild type and become replication-competent (Brenner, 1995; Coutelle et al., 1994; Curiel et al., 1996). Dose-dependent inflammation occurred after nasal (Knowles et al., 1995) or lung (Crystal et al., 1994) administration of the cystic fibrosis transmembrane conductance regulator (CFTR) cDNA conjugated with adenoviral vectors. The low efficacy, if any, is what one would have expected of pioneering studies. However, the risk to benefit ratio cannot be ignored. Also viruses produce antigens. When exposed to the host immune system, through leakage, secretion or cell damage, these antigens trigger immune reactions against the transduced cells. Certain viral elements are also toxic. These three inherent problems post almost insurmountable difficulties that prohibit the safe and efficacious clinical use of viral vectors at the present except for terminal cases. To raise caution, the FDA has mandated viral vector validation of every batch to be used on humans.

3. Plasmids

Single gene manipulation, often exercised *ex vivo*, has been used *in vivo*. Recombinant genes by themselves were shown to have been taken up and expressed in murine skeletal myofibers (Wolff et al., 1990; Ascadi et al., 1991; Davis et al., 1993) and cardiac myocytes (Leinwand and Leiden, 1991) following intramuscular injections. Gene expression is invariably low despite different delivery conditions and methods (Wolff et al., 1991). This approach lacks basis and evidence of gene integration and regulation.

4. Combinations

A more logical approach is to include viral or cellular transcriptional regulatory sequences to effect expression. In the prophylactic treatment of hemophilia A, a retroviral factor-VIII cDNA conjugate was used to induce secretion of the blood-clotting factor in athymic mice from transduced human skin fibroblasts implanted (Hoeben, 1995). Both adenoviral (Smith et al., 1993) and Herpes Simplex virus-derived (Miyanohara et al., 1992) vectors have similarly been used for *in vivo* transfer of factor IX cDNA to the liver. Although therapeutic levels of factor IX were obtained, the expression decayed in a few weeks, possibly due to immune response and gene inactivation (St. Louis and Verma, 1988).

Gene therapy with viral vectors has been developing rapidly, but judging from the results of cystic fibrosis and brain tumor clinical trials, it is still a young discipline (Rosenfeld and Collins, 1996; Alton and Geddes, 1994). Since the main thrust of this chapter is on myoblast transfer therapy (MTT), additional details of non-myoblastic single gene manipulations can be found in the books entitled "Gene Therapy - A Primer for Physicians" (Culver, 1996) "Somatic Gene Therapy" (Chang, 1994) and "Gene Therapy for Neoplastic Diseases" (Huber and Lazo, 1994).

5. Myoblasts

Although genetic ailments constitute less than 2% of all human diseases, far more currently incurable diseases are the result of inadequate genetic predisposition and/or haphazard interactions between multiple genes. Symptoms precipitate when a regulatory or a structural protein is either missing or malfunctional. Without knowing these defect(s) or how they can be corrected, tissue engineering will favor genome replacement rather than single gene(s) replacement. The cell knows more than we do.

Furthermore, for a gene therapy to be effective and efficient, transgene expression requires appropriate targeting into a specific cell type, integration onto a specific site on a specific chromosome, and regulation by factors that are the products of other genes. This chain of events involves numerous cofactors many of which are produced transiently during embryonic development but not in adulthood. This is where the approach of single gene manipulation is conceptually inadequate because it cannot provide these cofactors. In complex systems, one hardly knows what they are. Again only transfer of the whole normal genome can allow the orderly provision of these cofactors necessary for the transgene expression.

Finally secondary degenerative changes often accompany the primary protein defect. Additional structural and/or regulatory protein(s) are lost (**Fig. 1**). Even if single gene manipulation replaces the primary protein deficit, transduced cells still degenerate because of the secondary changes. These latter proteins can only be replaced by re-transcribing the normal genome inserted.

Myoblasts are muscle-building cells endogenous to the human body. Contained within the nucleus of each human myoblast is the normal genome with over 100,000 normal genes that determine cell normality and cell characteristics. Less than 10% of the gene actions is known. Myoblasts is the only somatic cell type in the body capable of natural cell fusion. Through this process, they insert their nuclei, and therefore all of the normal genes, into multinucleated myofibers of the host to effect genetic repair (**Fig. 2**).

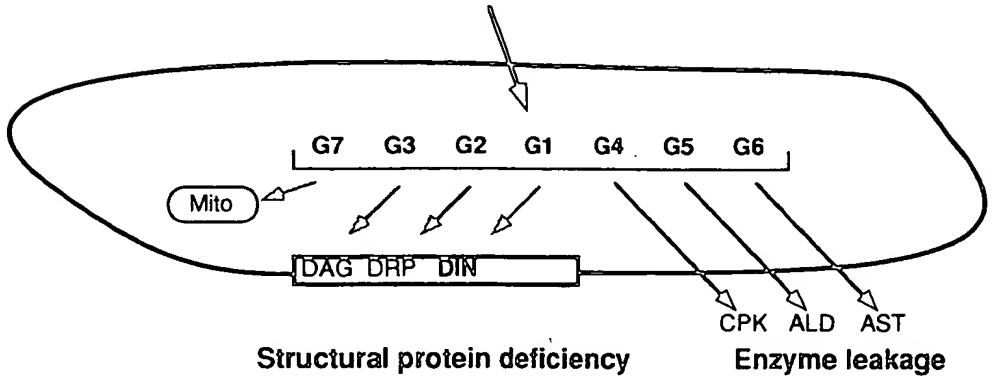


Fig. 1. Diagram of some of the known genetic factors in DMD muscle cells that differ from normal muscle cells. These include genes for membrane structural proteins that are decreased or absent in DMD, dystrophin (DIN), dystrophin-related-proteins (DRP) and dystrophin-associated glycoproteins (DAG), genes for enzymes elevated in serum levels of DMD patients, creatine phosphokinase (CPK), aldolase (ALD) and aspartate transaminase (AST), and genes for mitochondrial (Mito) differences.

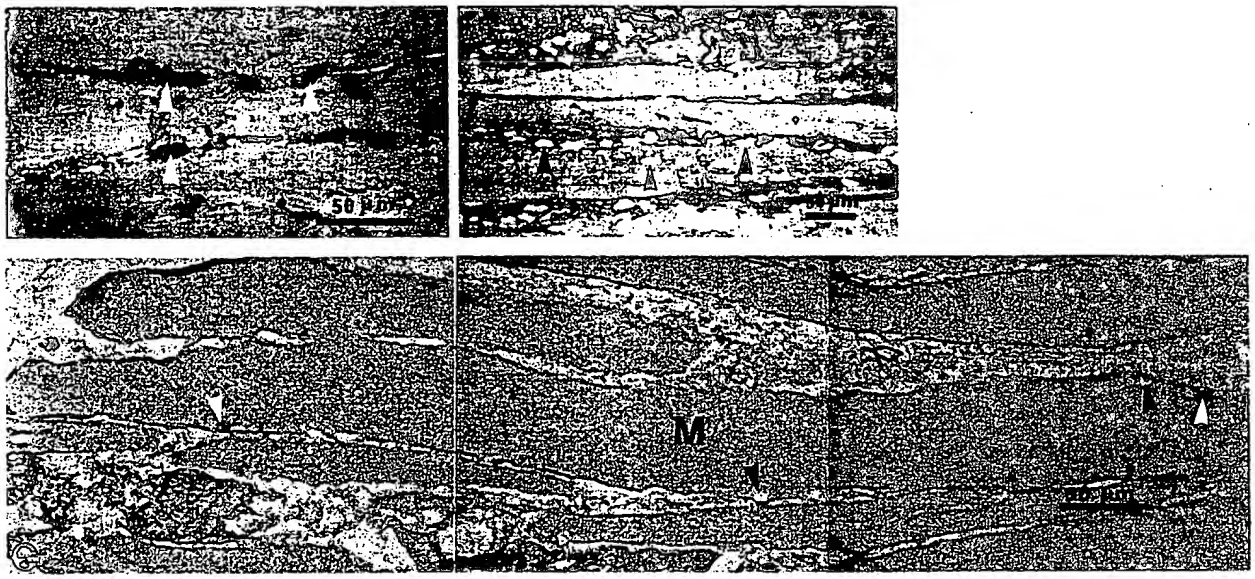


Fig. 2. Immunocytochemical localization of donor (stained, white arrowheads) and host (unstained, dark arrowheads) nuclei in longitudinal muscle sections. A and B are normal and dystrophic controls, respectively. C is from a dystrophic muscle 18 months after normal myoblast injection. A mosaic fiber (M) is demonstrated by the presence of both stained and unstained nuclei.

The transfer of genetic material and information occurs *in vivo*, with the myoblasts serving as the source and the vehicle to effect gene transfer.

Myoblasts are the only cells that divide extensively (Law et al., 1997a), migrate (Law et al., 1992), fuse naturally to form syncytia (Law et al., 1992), interiorizing major histocompatibility complex class I (MHC-1) antigens after fusion (Daar et al., 1984; Appleyard et al., 1985), and develop up to 50% of human body weight. Myoblast recipients need no more than two months of immunosuppression after MTT because mature myotubes and myofibers do not exhibit MHC-1 antigens (Daar et al., 1984; Appleyard et al., 1985). These combined properties render myoblasts superior for gene transfer. Being endogenous cells, myoblasts do not produce the adverse reactions of viral vectors.

II. Myoblast Transfer Therapy (MTT) technology

MTT is a platform technology of gene therapy and tissue engineering. The procedure consists of culturing large quantities of myoblasts from muscle biopsies of genetically normal human donors. Cultured myoblasts are

ient's muscles while the patient is under esia. An immunosuppressant is owing the procedure to minimize donor

injury activates regeneration of host wing them to fuse with the injected ius forming genetically mosaic yofibers (**Fig. 2**) (Law et al., 1988a,b, 92). In addition, injected myoblasts fuse es, forming genetically normal myofibers 88a,b; Chen et al., 1992). Thus, MTT mal nuclei, the genetic software and , into muscles of the genetically defective critical transgene is naturally and stably ed and expressed.

ion process is a natural occurrence, there any problem with specificities of plementation, regulation, and expression enome inserted. It is not necessary to ene(s) is responsible for the defect. identification is time-consuming and rmore, the injection of normal myoblasts ost muscle eliminates any uncertainty of Natural transcription of the normal ie donor nuclei following MTT ensures nt of any protein deficiency resulted from fects or from haphazard polygenic h of which is unknown.

lar dystrophies: the testing

strophies are genetic diseases of le degeneration. Debilitating and fatal, generative diseases deprive their sufferers lity of life and life span. Duchenne y (**DMD**) confines boys to wheelchairs laims their lives by 20. Second in o cystic fibrosis, DMD afflicts one in irths worldwide (Emery, 1991).

hereditary degenerative disease, DMD quire repairing degenerating cells and cells. MTT is unique in treating the hies in that it transfers the normal degenerative myofibers and it provides lenish degenerated myofibers. As such, ied cell/gene therapy. Potentially, not prevent further weakening, it can also rength.

strophy, DMD serves as a disease model cell/gene therapy in treating hereditary ses. MTT is being developed to repair and to replenish degenerated cells of the ie neuromuscular diseases affecting over e worldwide. In a broad sense MTT is ility, safety, and efficacy to integrate the ome into genetically abnormal patients.

Since MTT incorporates all of the normal genes into the dystrophic myofibers to repair them, it should exert similar effects regardless of which gene is abnormal or which protein is missing. Accordingly, MTT should be as beneficial to the murine dystrophies showing laminin α2 mutation in the dy and dy^{2J} phenotypes (Sunada et al., 1995) as DMD showing dystrophin deletion (Hoffman et al., 1987), given adjustments from mouse to human.

IV. Animal experiments

To develop a treatment we need to know the pathogenesis of the disease. By comparing the electric (Law and Atwood, 1972; Law et al., 1976) and ultrastructural properties (Mokri and Engel, 1975; Law et al., 1983) of normal vs. dystrophic myofibers, the genetic defects in muscular dystrophy were established to result from membrane deterioration and dysfunction. Using a normal/dystrophic parabiotic mice model with cross-reinnervation of muscles, it was demonstrated that the dystrophic nervous system would support normal muscle development (Law et al., 1976; Saito et al., 1983). Without such knowledge, it would be imprudent to attempt strengthening dystrophic muscles with normal myogenic cell transfer.

Earlier developmental work of MTT consisted of two approaches that were disparate but complementary. These are the demonstration of safety and efficacy of transferring normal myogenic cells into the $dy^{2J}dy^{2J}$ dystrophic mice (Law, 1978; Law and Yap, 1979; Law, 1982) and the examination of the developmental fate of donor cells in normal mice (Partridge et al., 1978; Watt, 1982; Watt et al., 1982). The $dy^{2J}dy^{2J}$ dystrophic mice share a common gene defect of laminin α2 mutation with congenital muscular dystrophy, the most severe form of human dystrophies (Sunada et al., 1995).

It was not until 1989 that a study of MTT on mdx mice was first published (Partridge et al., 1989; Karpati et al., 1989). The majority of evidence in support of MTT safety and efficacy is derived from previous studies using the $dy^{2J}dy^{2J}$ mice (Law et al., 1988a,b; Chen et al., 1992; Law, 1978; Law and Yap, 1979; Law, 1982; Law et al., 1990b,d).

This was at a time when neither the golden retriever muscular dystrophy (GRMD) nor the xmd canine dystrophy was known. Dystrophic dogs are available to a few laboratories that have not produced any significant results with MTT (Kornegay et al., 1992).

Central to MTT is the correlation of genetic and phenotypic improvement at the cellular and at the whole muscle levels. These studies play an essential role in the elucidation of the mechanisms by which MTT exerts its beneficial effects on dystrophic muscles (Law et al., 1978; Law and Yap, 1979; Law, 1982; Law et al., 1988a,b; Chen et al., 1992; Partridge et al., 1989; Karpati et al., 1989; Law et al., 1990b,d).

The demonstration that cultured cells survived, developed and functioned *in vivo* after implantation into an organ of a genetically abnormal mammal bridges the gap between *in vitro* and *in vivo* cell biology. This was first achieved with myoblast transfer (Law et al., 1988a,b).

The foremost study in adult *dystrophic* mice was aimed at producing mosaic muscles containing normal, dystrophic and mosaic myofibers from the *normal* and *dystrophic* minced muscle mixes (Law, 1978). It focused on incorporating the "missing" gene and its product(s) into genetically defective cells through cell transplantation and natural cell fusion, the result of which is strengthened dystrophic muscles (Law, 1978) having a gene defect similar to human congenital muscular dystrophy (Sunada et al., 1995). The result contradicts the study of Partridge and Sloper (1977) who concluded, in transplanting normal minced muscles into normal hosts, that little or none of the regenerates was of donor origin. Eventually, fusion between host and donor myogenic cells of normal genotypes using skeletal muscle grafts were demonstrated with genotype marker (Partridge et al., 1978). Although this latter study did not involve dystrophic animals, it was inferred that MTT was a distinct development with potential applicability to hereditary myopathies.

In a later study, muscles of newborn normal mice were grafted into recipient soleus muscles of dystrophic mice. Results obtained 6 months after the grafting indicated that the grafts survived, developed, and functioned in the dystrophic environment. The regenerates had larger cross-sectional areas and more muscle fibers than the contralateral dystrophic solei. MTT increased the mean twitch tension of adult dystrophic muscles to that of the normal (Law and Yap, 1979). The concept of replenishing lost cells and repairing degenerative cells through the production of genetic mosaicism using MTT was firmly established (Law and Yap, 1979).

An important finding was that myoblasts cultured from muscle biopsies of adult normal rats could survive and develop in the original donor after implantation (Jones, 1979). MTT with cultured myoblasts became the logical development since myoblasts do not require neuronal and capillary connections to survive and develop, and since myoblasts can fuse to effect genetic repair.

A convenient way to obtain normal myoblasts in mice is through dissection of limb-bud mesenchyme of day-12 embryos. Dissected mesenchyme was surgically implanted into the solei of $dy^{2J}dy^{2J}$ mice. Host and donors were histocompatible. Contralateral solei served as controls. Six to seven months postoperatively, the myoblast-implanted solei exhibited greater cross-sectional area, total fiber number, better cell structure, and twitch and tetanus tensions than their contralateral controls (Law, 1982).

The incorporation and fusion of allogeneic muscle precursor cells *in vivo* were further explored using normal mice (Watt, 1982). The implants consisted of minced muscle mixes or newborn muscles (Watt et al., 1982; Watt et al., 1984; Morgan et al., 1988). It was confirmed that donor cells survived and developed in the host muscles, using electrophoretic analyses of glucose phosphate isomerases (GPI), the genetic markers to identify hosts vs. donor cells.

The use of cultured myoblasts with dystrophic mice eventually appeared. In the first study, primary myoblast cultures from limb-bud explants of normal mouse embryos were injected into the soleus muscles of histocompatible dystrophic hosts (Law et al., 1988,b). In the second study, clones of normal myoblasts were injected into the leg and intercostal muscles of histoincompatible hosts with cyclosporine-A (**CsA**) as a host immunosuppresant (Law et al., 1988a). Using GPI as genotype markers, donor myoblasts were shown to have fused among themselves, developing into normal myofibers. They also fused with dystrophic host myogenic cells to form mosaic myofibers of normal phenotype (Law et al., 1988a,b; Law et al., 1990a,c). These two mechanisms of genetic complementation were shown to be responsible for improvement in muscle genetics, structure, function and animal behavior of the test dystrophic mice (Law et al., 1988a,b; Law, 1978; Law and Yap, 1979; Law, 1982; Law et al., 1990b,d). Prolongation of the life-spans of the myoblast-injected dystrophic mice was demonstrated (Law et al., 1990b,d). The improvement persisted despite CsA withdrawal.

Morgan *et al.* (1988) reported the synthesis of trace amounts of phosphorylase kinase (**PhK**) in about 5% of the myoblast-injected muscles of the PhK-deficient mice. Although there have been frequent claims of supplying normal muscle precursor cells to alleviate hereditary myopathies, no evidence of any structural or functional improvement after transplantation was presented.

With the discovery that the absence of the gene product dystrophin is the cause of DMD (Hoffman et al., 1987) and mdx mouse dystrophy, a new biochemical marker became available to demonstrate MTT efficacy (Partridge et al., 1989; Karpati et al., 1989; Chen et al., 1992). With implantation of cultured normal myoblasts into muscles of immunosuppressed mdx mice, MTT was shown to convert mdx myofibers from dystrophin-negative to -positive (Partridge et al., 1989; Karpati et al., 1989). The study demonstrates biochemical improvements in the mdx mouse model, an additional evidence to confirm the efficacy of MTT.

Given the use of inbred mice that afford histocompatible MTT, the reality is that fully matched human donors and dystrophic recipients are rarely available. MTT would thus necessitate the inclusion of host immunosuppression to facilitate myoblast survival after transfer. Cyclosporine (**Cy**) is the most widely documented immunosuppressant in transplantation studies (Kahan and Bach, 1988). Availability of FK506 in the late 80's was limited (Starzl et al., 1991). Typically, host mice were primed 1 week with CsA injected subcutaneously every day at 50 mg/kg body weight before receiving myoblasts. The same CsA treatment continued for 6 months after MTT (Law et al., 1988b).

Aside from donor cell survival in an immunologically hostile host, cell fusion is the key to strengthening dystrophic muscles with MTT. To improve the fusion rate between host and donor cells, various injection methods aimed at wide dissemination of donor myoblasts were tested and compared. The goal was to achieve maximum cell fusion with the least number of injections.

The results indicate that delivery of myoblasts is best conducted by diagonal placement of needle into the host muscle with ejaculation of the myoblasts as the needle is withdrawn. This method of myoblast injection yields even and wide distribution of donor myoblasts with a high rate of cell fusion. Myoblasts injected perpendicular to myofiber orientation are partially distributed. Myoblasts injected longitudinally through the core of the muscles and parallel to the myofibers are poorly distributed (Law et al., 1994b). Thus myoblast injection method regulates cell distribution and fusion.

V. Clinical trials

Gene therapy encompasses interventions that involve deliberate alteration of the genetic material of living cells to prevent or to treat diseases (Kessler et al., 1993). According to this FDA definition, the first MTT on a DMD boy on February 15, 1990 marked the first clinical trial on human gene therapy (Hooper, 1990). In addition to fulfilling their primary muscle-building mission, the myoblasts served as the source and the transfer vehicles of normal genes to correct the gene defects of DMD. The protocol was approved by four institutional review boards (Law, et al., 1990c). Subjects and parents gave informed consents.

The safety and efficacy of MTT was assessed by injecting the left extensor digitorum brevis (EDB) muscle of a 9-yr-old DMD boy with about 8×10^6 myoblasts. Donor myoblasts were cloned from satellite cells derived from a 1 g rectus femoris biopsy of the normal, adoptive father. Cyclosporine was administered for three months at a dose of 5-7 mg/kg body weight divided into two daily oral doses.

Donor myoblasts survived, developed, and produced dystrophin in myofibers biopsied from the myoblast-injected EDB 92 days later. Dystrophin was not found in the contralateral sham-injected muscle. This first case suggested that MTT offered a safe and effective means for alleviating biochemical deficit(s) inherent in muscles of DMD (Law et al., 1990a).

A pioneering work (Anderson, 1990; see also Brenner, 1995; Karlsson, 1991) is often considered as the "first human gene therapy"; correction of the ADA deficiency study began on September 14, 1990 (Anderson, 1990), two months after the MTT correction of the DMD gene defect was published (Law et al., 1990a). In the ADA protocol, T cells from a patient with a severe combined immunodeficiency disorder (SCID) were transduced with functional ADA genes *ex vivo* and returned to the patient after expansion through culture. In the MTT protocol, primary culture of myoblasts derived from a muscle biopsy of a normal donor was injected into a muscle of the DMD subject to produce *in vivo* nuclear complementation. Both gene therapies utilize cell transplantation to treat diseases.

However, it is pointed out that the ADA protocol involved genetic modification and correction of the patients T cells with the *adenosine deaminase* gene whereas in the DMD protocol normal donor cells were used which were not genetically modified ex vivo.

Six years after the foremost MTT, dystrophin was found in the myoblast-injected muscle but not in the sham-injected muscle (**Figure 3**, Law, 1997). Six years is the longest period through which any gene therapy has sustained positive results. Despite cyclosporine withdrawal at 3 months after MTT, myofibers expressing foreign dystrophin were not rejected. This is because dystrophin is located in the inner surface of the plasma membrane, and because mature myofibers do not exhibit MHC-1 surface antigens. Not only has the result demonstrated MTT overall safety and efficacy in this single case, it also shows stability in the integration, regulation and expression of the inserted dystrophin gene. The presence of dystrophin in the myoblast-injected but not in the sham-injected muscle provided unequivocal evidence of the survival and development of donor myoblasts in the myoblast-injected muscle.

In a randomized double-blind study involving three subjects, myoblast-injected EDBs showed increases in tensions whereas sham-injected EDBs showed reductions (Law et al., 1991a,b). Both immunocytochemical staining and immunoblot revealed dystrophin in the myoblast-injected EDBs. Dystrophic characteristics such as fiber splitting, central nucleation, phagocytic necrosis, variation in fiber shape and size, and infiltration of fat and connective tissues were less frequently observed in these muscles. Sham-injected EDBs exhibited significant structural and functional degeneration and no dystrophin. Throughout the study, there was no sign of erythema, swelling or tenderness at the injection sites. Serial laboratory evaluation including electrolytes, creatinine, and urea did not reveal any significant changes before or after MTT.

To reconcile these positive results with less convincing ones (Gussoni et al., 1992; Huard et al., 1992; Karpati et al., 1993; Mendell et al., 1995; Miller et al., 1992; Morandi et al., 1995; Tremblay et al., 1993), several issues need to be addressed. To begin with, the use of large quantities of pure live myoblasts is a pre-requisite of successful MTT. Except for one study (Law et al., 1992), there is no published pictorial evidence to substantiate the purity, myogenicity and viability of the injected myoblasts as claimed.

Myoblast cultures are usually contaminated with fibroblast overgrowth. MTT with such impure culture could lead to deposition of connective tissues rather than myofiber production. Culturing 50 billion pure human myoblasts for MTT from two grams of muscle biopsy has

only been reported by our team (Law et al., 1997a). Other teams work at ranges of hundreds of millions of myoblasts.

In most studies (Gussoni et al., 1992; Karpati et al., 1993; Mendell et al., 1995; Miller et al., 1992; Morandi et al., 1995) myoblasts were transported frozen, chilled for over two hours from the site of harvest before being injected. Since myoblasts have a high metabolic rate, they could not have survived for two hours without significant nutrients, oxygen and proper pH, being closely packed in saline within a vial for transport. Determination of cell viability before MTT were not conducted in these studies. Our myoblasts were injected into the subject within minutes of harvest, at the same location without transport.

MTT studies that reported failure (Gussoni et al., 1992; Huard et al., 1992; Karpati et al., 1993; Mendell et al., 1995; Miller et al., 1992; Morandi et al., 1995; Tremblay et al., 1993) subscribed to the fallacy of making 55 to 330 injections into a muscle the size of an egg, traumatizing indiscriminately the underlying nerves, muscle, and vasculature. These injection traumas boosted macrophage access and host immune responses (Guerette et al., 1995). They also induced fibrosis (Chen et al., 1988). Surviving myoblasts fused within three weeks in small mouse muscles (Chen et al., 1992). A nerve with multiple trauma could not regenerate soon enough through scar and connective tissues to innervate the newly-formed myotubes in a large human dystrophic muscle. Stabilization of muscle contractile properties in a similar situation is achieved by 60 days in the rat, and functional return is incomplete (Carlson, 1983). Non-innervated myotubes died within one week. Whatever few myotubes that developed in the unsuccessful MTT studies could not compensate for the traumatized myofibers.

In the study yielding positive results, 5 to 8 x 10^8 pure myoblasts were delivered with eight injections into the biceps brachii without nerve injury (Law et al., 1994a, 1997a). Contrarily, in another study, 55 sites, each 5 mm apart, distributed in 11 rows and 5 columns, were injected throughout the depth of each biceps of 5- to 9- year old boys (Mendell et al., 1995). This was repeated monthly for six months. Axonal sprouts, myotubes and neuromuscular junctions that take six weeks to mature (Fex and Jirmanova, 1969) were repeatedly traumatized by a total of 330 injections until the biceps, with or without myoblast/cyclosporine, were irreversibly damaged or destroyed. The result: no functional difference between myoblast- and sham-injected muscles (Mendell et al., 1995).

Once injected, the myoblasts are subjected to scavenger hunt by macrophages for up to three weeks. This is because myoblasts exhibit MHC-1 surface antigens (Friedlander and Fischman, 1979; Fang et al., 1994) that become absent after cell fusion. The latter occurs between one to three weeks after myoblast injection (Chen et al., 1992). An allowance in the number of injected myoblasts has to be made to satisfy the unavoidable scavenger process. As reflected in the small numbers of myoblasts injected in unsuccessful studies, it appears that either such allowance was not considered or that the teams were not able to produce larger quantities of pure myoblasts. Although myoblast loss can be minimized by down-regulating macrophage activity (Guerette et al., 1997), such additional compromisation of the host immune system may lead to higher risk of infection, since MTT subjects are already taking immunosuppressants.

The less successful MTT teams focused on immunosuppression to prevent T-lymphocyte proliferation and antibody production without overcoming the primary hurdle of providing enough pure and live myoblasts. A basic study indicates that cyclophosphamide did not permit myoblast engraftment in the mouse (Vilquin et al., 1995), and a MTT clinical trial was conducted without success using cyclophosphamide immunosuppression (Karpati et al., 1993). Cyclosporine (Law et al., 1990a) and potentially FK506 (Kinoshita et al., 1994) remain the immunosuppressants of choice for MTT. Results could have been more positive if either was employed in the study of Tremblay *et al.* (Huard et al., 1992; Tremblay et al., 1993).

All of these single muscle MTT studies had begun before the FDA established policies and regulations for cell/gene therapies. Our studies are the only ones that received permission for an investigational new drug application (IND) on MTT for treatment of multiple muscles. As a cell/gene therapy, all American MTT clinical trials must come under FDA purview.

Beginning with 8 million myoblasts into a small foot muscle, our team proceeded to test 5 billion cells into 22 leg muscles, 25 billion cells into 64 body muscles, and now 50 billion cells into 82 muscles (**Table 1**). With over 150 procedures having been conducted, the complete safety of the MTT procedure has been proven. There have been no adverse reactions or side effects.

Protocol	Myoblasts	Muscles	Subjects
1	8 million	1	11
2	5 billion	22	32
3	25 billion	64	40
4	50 billion	82	27

Table 1. Dose escalation protocols of MTT and the number of subjects receiving such procedures.

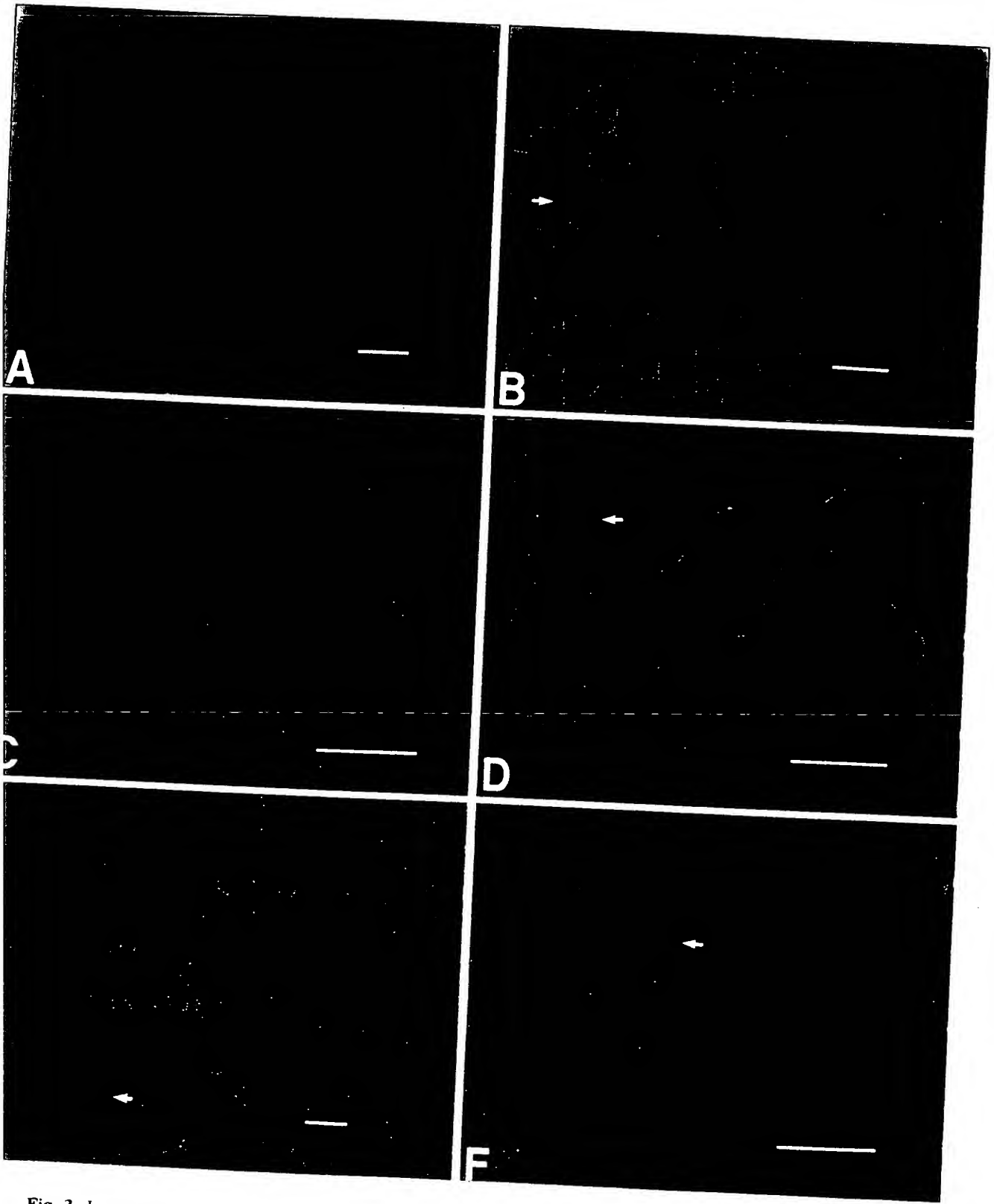


Fig. 3. Immunocytochemical demonstration of dystrophin in DMD muscles 6 yr after MTT. Dystrophin absent in sham-injected EDB muscle (**A**,**C**), but present in the contralateral myoblast-injected muscle (**B**,**D**). Dystrophin was immunocytochemically localized at the sarcolemma (arrows). Dystrophin demonstrated at low (**E**) and high (**F**) magnification in normal control muscle. Cross-section; bar = 100μm.

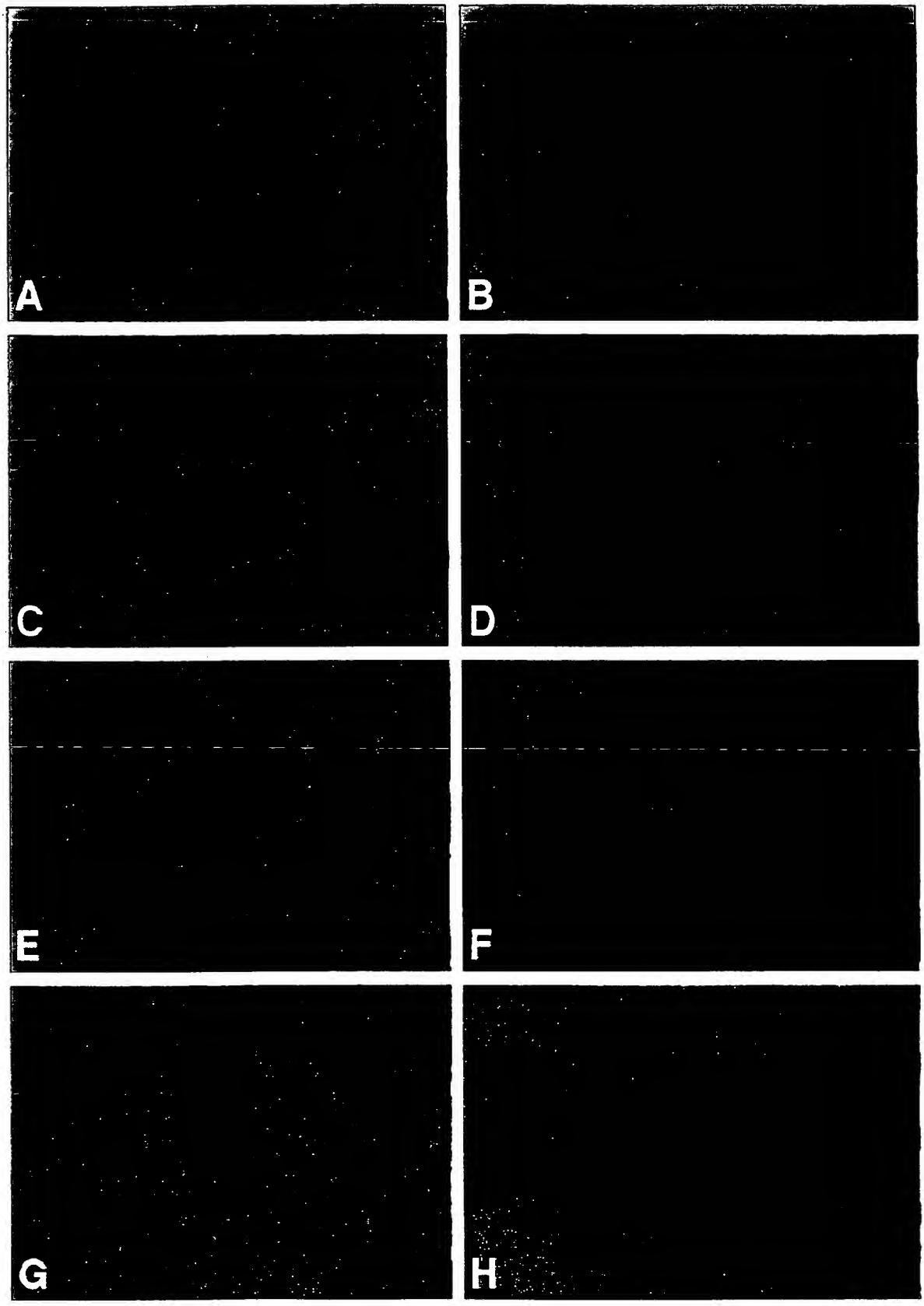


Fig. 4. Dystrophin immunocytochemistry showing the presence of dystrophin in (A) normal control and in (C,E,G) muscle biopsy specimens of three subjects. Dystrophin is absent in (B) Duchenne's muscular dystrophy control and in (D,F,H) contralateral biopsy specimens from the same subjects.

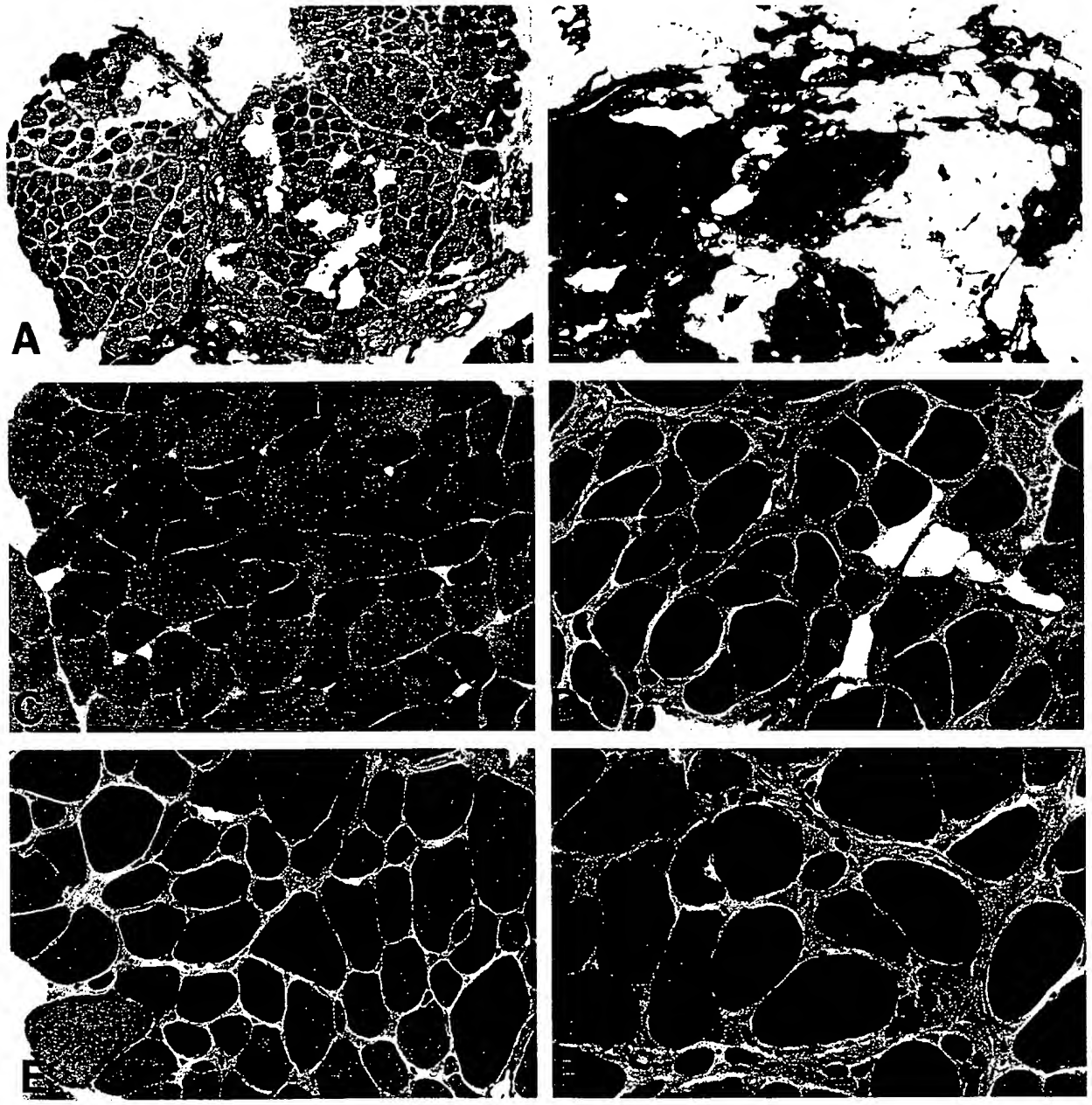


Fig. 5. (**A,C,E**) Three dystrophin-positive muscle biopsy specimens exhibit less dystrophic characteristics than the contralateral dystrophin-negative biopsy specimens (**B,D,F**). Dystrophic characteristics include increases in fat and connective tissue, fiber splitting, central nucleation, round and oval fibers.

The five billion myoblast cell protocol.

The 5-billion myoblast MTT protocol was tested in 32 DMD boys aged 6-14 yr. Through 48 injections, 5 billion myoblasts were transferred into 22 major muscles in both lower limbs under general anesthesia. Only four donors were histocompatible with their recipients. All subjects took cyclosporine for six months after MTT. More than 88% of the injected ankle plantar flexor muscles showed either increase in strength or no further deterioration at 9 months after MTT (Law et al., 1992, 1993).

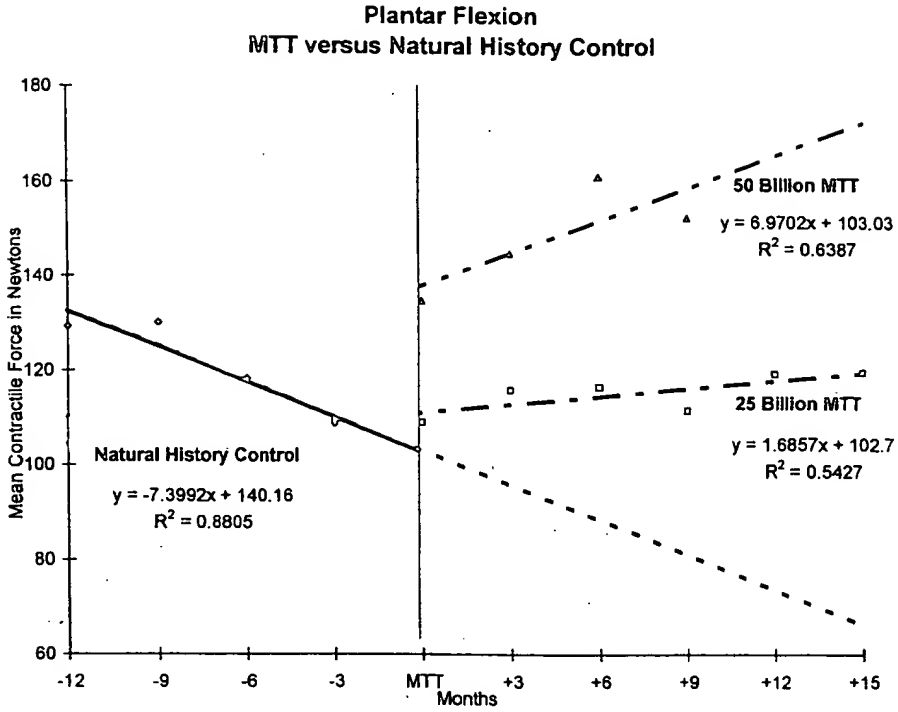


Fig. 6. Dose-dependent responses to MTT of plantar flexion with greater increase in maximum isometric force using the 50-billion MTT protocol than with the 25-billion MTT protocol. Both protocols show efficacy in strengthening the plantar flexion when compared to the natural history control.

	Months after MTT				
	3	6	9	12	15
25 Billion MTT (800 x 10^6 myoblasts)	9%	19%	31%	45%	61%
50 Billion MTT (1,200 x 10^6 myoblasts)	11%	23%	37%	53%	71%

Table 2. Percentage increases over a one-year natural history control in the maximum isometric force of the plantar flexor muscles at 3, 6, 9, 12, and 15 months after the administration of the 25-billion MTT protocol or the 50-billion MTT protocol.

The 25 billion myoblast cell protocol.

Under FDA purview, MTT is completing Phase II clinical trials on DMD. The whole body trial (WBT) consisted of injecting 25 billion myoblasts in two MTT procedures separated by 3 to 9 mo. Each procedure delivered up to 200 injections or 12.5 billion myoblasts to either 28 muscles in the upper body (UBT) or to 36 muscles in the lower body (LBT). A randomized double-blind portion of the study was conducted on the biceps brachii or quadriceps. Subjects took oral cyclosporine for 3 months after each MTT. One infantile facioscapulohumeral dystrophy and 40 DMD boys aged 6 to 16 received WBT in the past 36 months with no adverse reaction.

Nine months after MTT immunocytochemical evidence of dystrophin were demonstrated in 18 of the 20 DMD subjects biopsied (**Fig. 4**). Dystrophin positive sections showed less dystrophic characteristics than dystrophin-negative ones (**Fig. 5**). Forced vital capacity increased by 33.3% and maximum voluntary ventilation increased by 28% at 12 months after UBT (Law et al., 1997a).

Plantar flexion showed an increase of 45% in maximum isometric contraction force in 12 months in the DMD subjects when compared to the natural deterioration (**Fig. 6, Table 2**). Behavioral improvements in running, balancing, climbing stairs and playing ball were noted (Law et al., 1995; Law et al., 1996; Law et al., 1997a,c,d). Notable was a 16-yr-old DMD subject who continued to walk without assistance and capable of driving an automobile by himself.

50 Billion myoblast cell protocol.

The current study involves a one time injection of 50 billion myoblasts into 82 muscles with 179 skin punctures, approved by the FDA for subjects with DMD, Becker MD and Limb-girdle MD (Law et al., 1997d). Twenty-nine subjects who underwent this protocol have experienced no adverse reaction.

For the 22 DMD subjects aged 5 to 16, there was a significant increase in the maximum isometric force generated by the plantar flexor muscles at 3, 6, and 9 months after MTT (**Fig. 6, Table 2**).

This functional improvement is more pronounced with the 50-billion MTT than with the 25-billion MTT, indicating that it is dose-dependent. Thus, in the 25-billion MTT, 800 million myoblasts were injected into the plantar flexors, producing a mean 61% increase in force at 15-months after MTT. With the 50 billion MTT, 50% more myoblasts were injected, projecting a 10% greater increase in force at 15 months after MTT (**Fig. 6, Table 2**).

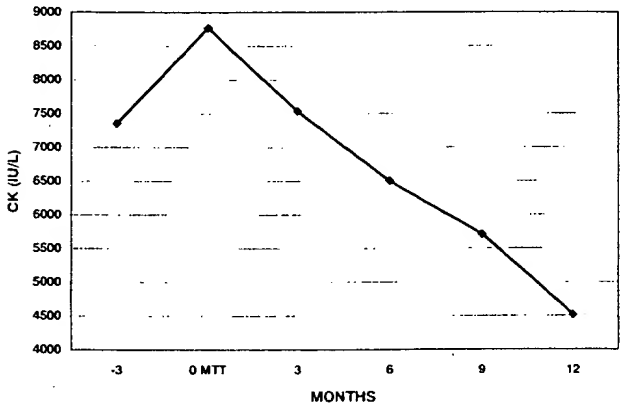


Fig. 7. Serum creatine kinase (CK) level of DMD subjects increased before MTT and decreased after MTT.

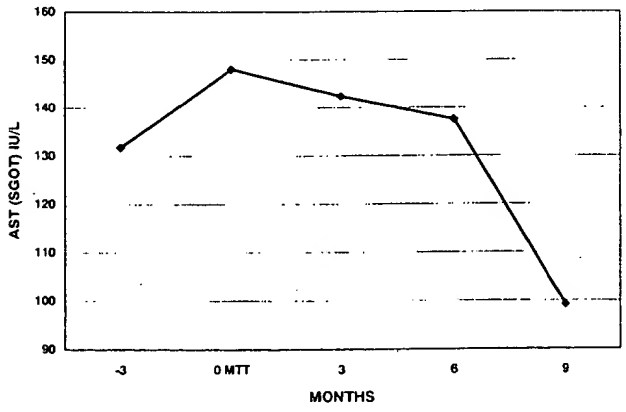


Fig. 8. Serum aspartate aminotransferese (AST) level of DMD subjects increased before MTT and decreased after MTT.

Elevated serum creatine kinase (**CK**) has traditionally been used to diagnose muscle degeneration, notable in DMD (Heyck et al., 1966). The 22 DMD subjects, mean ages 10.7-yr-old and, median age 9.9 yr-old, showed a 19.3% increase in serum CK within 3 months before MTT (**Fig. 7**). This trend was reversed after MTT, and the serum CK declined at a steady rate of 48.7% over 12 months. This result provides strong evidence that MTT repairs muscle cell membrane leakage of enzymes. This contention is further substantiated by similar findings with another muscle enzyme AST, aspartate aminotransferase (**Fig. 8**).

The breakthrough came when a 29-yr-old Becker MD (BMD) subject began to walk, with his hands being held, beginning at 2.5 months after the 50-billion MTT. He had previously been diagnosed repeatedly with BMD. He had been non-ambulatory and required the use of a wheelchair for over four years as documented in his medical record. He began walking with assistance a total of eight steps at 3 months after MTT. This ability increased with time, now reaching 60 steps at eight months after MTT. He began to stand and walk with his crutches at four months after MTT (**Fig. 9**).

VI. Future perspectives

As an universal gene transfer vehicle with which the entire human genome can be integrated into patient's muscles, myoblasts have shown promise in studies of the following diseases:

Cardiomyopathy. Labeled cultured myoblasts engrafted and formed structures resembling desmosomes, intercalated discs, fascia adherents junctions, and gap junctions in myocardia of dogs (Chiu et al., 1995), rats (Murry et al., 1996) and mice (Robinson et al., 1996) when MTT was delivered intramuscularly (Chiu et al, 1995; Murry et al., 1996) or intraarterially (Robinson et al., 1996). Donor muscle regenerates exhibited cardiac-like properties such as central nucleation (Chiu et al., 1995), fatigue resistance, slow twitching, and were capable of twitch and tetanus contractions when stimulated (Murry et al., 1996). Similar results were obtained when cardiomyocytes were injected in dystrophic mice and dogs (Koh et al., 1995), rats (Li et al., 1996) and swine (Van Meter et al., 1995). These findings, together with established MTT safety, pave the way to MTT clinical trial in treating myocardial degeneration and dysfunction.

Insulin-resistant diabetes mellitus. Commonly known as Type II diabetes, this disease is genetically predisposed and afflicts 90% of the diabetic population. Virtually all identical siblings of these patients develop the disease, and the genetic defect can be traced to the GLUT4 gene deletion. The major sequela of insulin resistance is decrease muscle uptake of glucose, due to the moderate decrease in insulin receptors on muscle cell surface. Conceptually MTT can add genetically normal myofibers with normal insulin receptors. It can also genetically repair the patients' myofibers and produce normal insulin receptors on the heterokaryons. Basic research is need to test this hypothesis on diabetic rats.

Fig. 9. First muscular dystrophy subject ever to walk after wheelchair bound for years.

(A). The 29-yr-old BMD subject had been wheelchair-bound for over 4 years.

(B,C,D,E) He began to walk with his hands held at 2.5 months after the 50-billion MTT.

(F) At 4 months after MTT, he was able to walk on crutches for about 20 steps.

Bone/cartilage degeneration. During embryonic development, mesenchymal progenitor cells differentiate into myoblasts, osteoblasts, chondrocytes and adipocytes under controls of various regulatory factors. Ectopic bone formation in muscle has been achieved through implantation of bone morphogenetic protein (BMP). BMP-2 was shown to convert the differentiation pathway of clonal myoblasts into the osteoblast lineage (Katagiri et al., 1994). This opens new ways to treat conditions of bone degeneration such as the degeneration of tooth pulp, hip, bone/joint, and long bone fractures. Given the ability to mass-produce myoblasts that can be transformed into osteoblasts, and potentially chondrocytes, the difficulty of proliferating osteoblasts and chondrocytes can be overcome. Cultured autologous chondrocytes can be used to repair deep cartilage defects in the femorotibial articular surface of the human knee joint (Brittberg et al., 1994).

The use of normal or transduced myoblasts as the source and vehicles for gene delivery has found application in the potential treatments of restenosis (Morishita et al., 1995), soft tissue deformities (Teboul et al., 1995), hemophilias (Dai et al., 1992; Yao et al., 1994), anemia (Hamamori et al., 1994), muscle trauma (Almeddine et al., 1994), human growth hormone deficiency (Barr and Leiden, 1991) and allograft rejection (Lau et al., 1996). MTT has produced a new frontier in medicine.

VII. Our vision

MTT implementation can benefit from the development of the following programs (Law, 1994):

Controlled cell fusion. It will be useful to be able to control, initiate or facilitate cell fusion once myoblasts are injected. This is to minimize loss of myoblasts from macrophages whose presence is unavoidable if the patient is to have some immune protection.

As the myoblasts are injected intramuscularly into the extracellular matrix, injection trauma causes the release of basic fibroblast growth factor (bFGF) and large chondroitin-6-sulfate proteoglycan (LC6SP). These latter growth factors stimulate myoblast proliferation. Unfortunately, they also stimulate the proliferation of fibroblasts that are already present in increased amount in the dystrophic muscle. That is why it is necessary to inject as pure as possible fractions of myoblasts in MTT without contaminating fibroblasts.

Controlled cell fusion can be achieved by artificially increasing the concentration of LC6SP over the endogenous level. In addition, insulin or insulin-like growth factor I (IGF-1) may facilitate the developmental process, resulting in the formation of myotubes soon after myoblast injection. Enhanced fusion of myoblasts into myotubes had been achieved with the use of PDO98059 (Coalican et al., 1997) and ED2+ macrophages conditioned medium (Massimino et al., 1997). The use of these compounds in the cell culture medium and in the injection medium will likely lead to greater MTT success.

Superior cell lines. These cell lines should be highly myogenic, nontumorigenic, nonantigenic, and will develop very strong muscles. The superior cell lines will bypass the use of immunosuppressant, and will provide a ready access for patients who do not have a donor. A unique property of myoblasts is their loss of MHC-I antigens soon after they fuse. The immunuosuppression period depends on how soon the myoblasts lose their MHC-I antigens after MTT. Even more ideal is the establishment of a myoblast cell line in which MHC-I antigens are absent. In human myoblasts cultured from normal muscle biopsies, some 91.7% of the myoblasts reacted with MHC-I MAb (monoclonal antibodies). The remaining 8.3% of the myoblasts, that were negative for MHC-I antigen expression were successfully separated by cytofluorometry. The lack of MHC-I antigens on these latter myoblasts may enhance survival of these myoblasts in recipients after MTT (Fang et al., 1994).

Automated cell processors. The great demand for normal and transduced myoblasts, the labor intensiveness and high cost of cell culturing, harvesting and packaging, and the fallibility of human imprecision will soon necessitate the invention and development of automated cell processors capable of producing huge quantities of viable, sterile, genetically well-defined and functionally demonstrated biologics.

This invention will be one of the most important offspring of modern day computer science, mechanical engineering and cytogenetics. The intakes will be for biopsies of various human tissues. The computer will be programmed to process tissue(s), with precision controls in time, space, proportions of culture ingredients and apparatus maneuvers. Cell conditions can be monitored at any time during the process and flexibility is built-in to allow changes. Different protocols can be programmed into the software for culture, controlled cell fusion, harvest and package. The outputs supply injectable cells ready for cell therapy or shipment. The cell processor will be self-contained in a sterile enclosure large enough to house the hardware in which cells are cultured and manipulated.

Transport medium. A transport medium that can sustain the survival and myogenicity of myoblasts in package for up to four days will allow the cell packages to be delivered to remote points of utilization around the world. **Fig 10** shows the effectiveness of such a medium developed in our foundation. Fifty billion myoblasts can be shipped at 4° C for four days with 90% viability.

Cell banks. The automated cell processors will constitute only a part of the cell banks. The current thought is to obtain donor muscle biopsies from young adults aged 8 to 22 to feed the inputs. Each donor has to undergo a battery of tests that are time-consuming and expensive. From the test results and from the donor's physical conditions, one can determine if the donor cells are genetically defective or infected with viruses and/or bacteria.

Human fetal tissues can potentially provide greater supplies of cells. However, aside from ethical issues surrounding abortion, it is difficult to determine the genetic normality of the cells. Muscle primordia of fetus derived from *in vitro* fertilization of genetically well-defined background may be an alternative. Sperm and ova can be recovered from healthy individuals that are known for their muscle strength and mass. *In vitro* fertilization will be followed by embryo culture to a specific developmental stage (day 26 to day 56 gestation) of the embryos. The muscle primordia that are rich in myoblasts can then be dissected out to feed the automated cell processors.

VIII. Conclusion

This chapter describes the landmark development of the first gene therapy study in humans. Through natural cell fusion, myoblasts transfer the human genome into dystrophic muscle cells to effect phenotype repair. The innovative cell transplantation procedure also revitalizes the degenerative organ by providing living cells of normal genotype to replenish cell loss. The result is potentially a new form of medicine. The conceptual approaches of single gene transfer and myoblast transfer toward treatment of hereditary degenerative diseases are compared.

As more scientists continue to recognize myoblasts as a stable source of genes and a safe and efficient gene transfer vehicle, MTT application will extend far beyond the treatment of neuromuscular diseases. This chapter provides insights to guide future development of MTT in battling against genetic and acquired diseases that presently have only diagnoses but no treatment.

Acknowledgment

Clinical trials are supported by public donations with FDA approval for direct cost recovery.

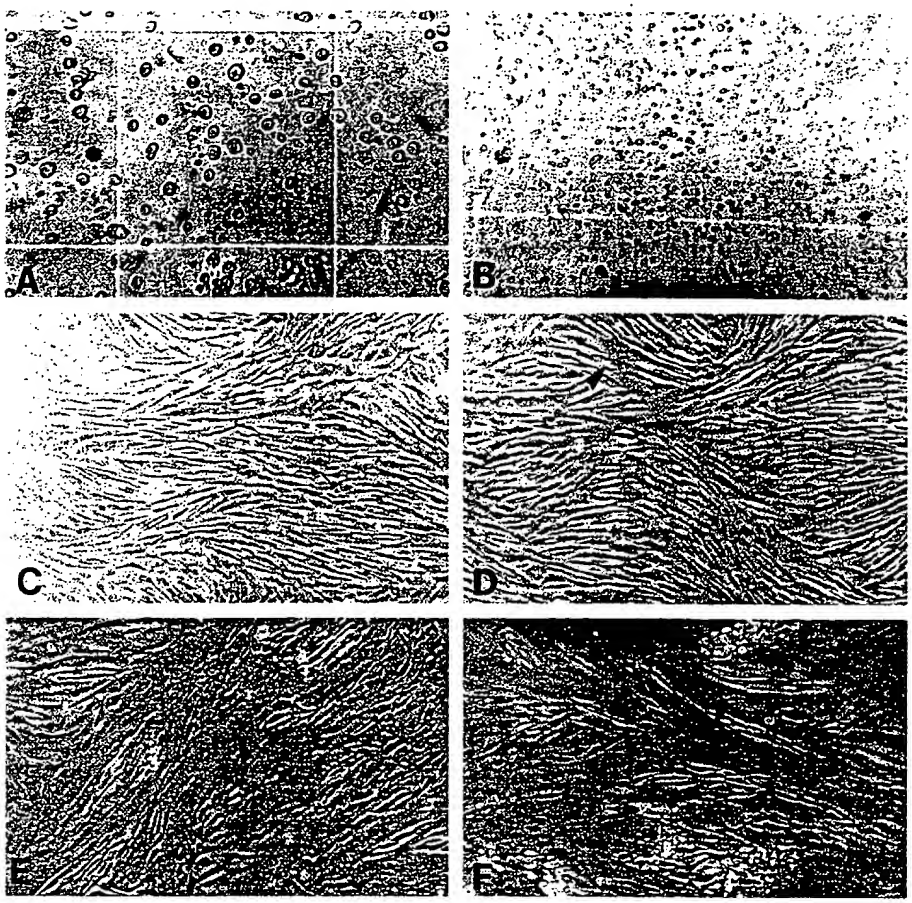


Fig. 10. Transport medium effectiveness as demonstrated by myoblast survival and myotube formation. (**A**). Myoblasts before a 50-billion MTT showing 99% viability using the vital stain erythrocin B,1% at pH 7.23. (**B**). Myoblast left-over from a 50-billion MTT maintained in the transport medium for 4 days at 4° C and stained with erythrocin B. The sample showed 90% viability. (**C**). Cells in B were put back into culture for 2 days before feeding fusion medium. (**D**). Cells in C in fusion medium for 1 day, showing myoblast fusion (arrow). (**E**). Cells in C in fusion medium for 2 days, showing myotubes (arrow). (**F**). Cells in C in fusion medium for 5 days, showing extensive myogenic capability in myotube formation (arrows).

References

Alameddine, H.S., Louboutin, J.P., Dehaupas, M. *et al.* **(1994)**. Functional recovery induced by satellite cell grafts in irreversibly injured muscles. **Cell Transplantation** 3, 3-14.

Albert, N., Tremblay, J.P. **(1992)**. Evaluation of various gene transfection methods into human myoblast clones. **Transpl. Proc.** 24, 2784-2786.

Alton, E.W.F.W. and Geddes D.M. **(1994)**. Gene therapy for cystic fibrosis , a clinical perspective. **Gene Therapy** 2, 88-95.

Anderson, W. F. **(1990)**. September 14, 1990 , The Beginning. **Hum. Gene Therapy** 1, 371-372.

Anderson, W.F. **(1992)**. Human Gene Therapy. **Science** 256, 808-813.

Anderson, W. F. **(1995)**. Gene Therapy. **Scient. Amer.** 273, 96-98B.

Appleyard, S. T., Dunn, M. J., Dubowitz, V.*et al.* **(1985)**. Increased expression of HLA ABC class I antigens by muscle fibres in Duchenne muscular dystrophy, inflammatory myopathy, and other neuromuscular disorders. **Lancet** 1, 361-363.

Ascadi, Gyula, Dickson, G. Loves, D.R. *et al.* **(1991)**. Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs. **Nature** 352, 815-818.

Barr, E., Leiden, J.M. **(1991)**. Systemic delivery of recombinant proteins by genetically modified myoblasts. **Science** 254, 1507-1509.

Brenner, M.K. (1995). Human somatic gene therapy , progress and problems. **Int. Med.** 237, 229-239.

Brittberg, M., Lindahl, A., Nilsson, A. *et al.* **(1994)**. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. **N. Engl. J. Med.** 331, 889-895.

Carlson, B.M. **(1983)**. The regeneratation and transplantation of skeletal muscle. In , Seil, F., ed. **Nerve, organ, and tissue regeneration, Research perspectives.** Academic Press. New York. pp. 431-454.

Chang, P. L. (ed.). **(1994)**. **Somatic Gene Therapy.** CRC Press. New York.

Chen M., Li, H.J., Fang, Q. *et al.* **(1992)**. Dystrophin cytochemistry in mdx mouse muscles injected with labeled normal myoblasts. **Cell Transplantation** 1, 17-22.

Chen, S.S., Chien, C.H., Yu, H.S. **(1988)**. Syndrome of deltoid and/or gluteal fibrotic contracture; an injection myopathy. **Acta Neurol. Scand.** 78, 167-176.

Chiu, R.C.J., Zibaitis, A., and Kao, R.L. **(1995)** . Cellular cardiomyoplasty , myocardial regeneration with satellite cell implantation. **Ann. Thorac. Surg.** 60, 12-18.

Coolican, S.A., Samuel, D.S., Ewton, D.Z. et al., **(1997)**. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. **J. Biol. Chem.** 272, 6653-6662.

Cornetta, K., Morgan, R.A., Anderson, W.F. **(1991)**. Safety issues related to retrovirus-mediated gene transfer in humans. **Hum. Gene Ther.** 2, 5-14.

Coutelle, C., Caplen, N., Hart, S. *et al.* **(1994)**. Towards gene therapy for cystic fibrosis. In , Dodge, J.A., Brock, J.H., and Widdicombe, J.H., eds. **Cystic Fibrosis , Current Topics.** John Wiley and Sons. New York. 2, 33-54.

Crystal, R.G., McElvaney, N.G., Chu, C.S. *et al.* **(1994)**. Administration of an adenovirus containing the human CFTR c DNA to the respiratory tract of individuals with cystric fibrosis. **Nature Genet.** 8, 42-51.

Culver, K. W. **(1996)**. **Gene Therapy, A Primer for Physicians.** Mary Ann Liebert, Inc.. Larchmont.

Curiel, D. T., Pilewski, J. M., Albelda, S.M. **(1996)**. Gene therapy approaches for inherited and acquired lung diseases. **Am. J. Respir. Cell Mol. Bol.** 14, 1-18.

Daar, A. S., Fuggle, S. Y., Fabre, J. W. *et al.* **(1984)**. The detailed distribution of HLA-A, B, C, antigens in normal human organs. **Transplantation** 38, 287-298.

Dai, Y., Roman, M., Naviaus, R.K. *et al.* **(1992)**. Gene therapy via primary myoblasts , long term expression of factor IX protein following transplantation *in vivo*. **Proc. Natl. Acad. Sci. USA** 89, 10892-10895.

Davis, H.L., Whalen, R.G., Demeneix, B.A. **(1993)**. Direct gene transfer into skeletal muscles *in vivo* , factors affecting efficiency of transfer and stability of experssion. **Hum. Gene Ther.** 4, 151- 159.

Emery, A.E. **(1991)**. Population frequencies of inherited neuromuscular diseases - a world survey. **Neuromus. Disord.** 1, 19.

Fang, Q., Chen, M., Li, H.J. *et al.* **(1994)**. MHC-1 antigens on cultured human myoblasts. **Transpl. Proc.** 26, 3467.

Fex, S. and Jirmanova, I. **(1969)**. Innervation by nerve implants of "fast" and "slow" skeletal muscles of the rat. **Acta Physiol. Scand.** 76, 257-269.

Friedlander, M. and Fischman, D.A. **(1979)**. Immunological studies of the embryonic muscle cell surface. Antiserum to the prefusion myoblast. **J. Cell Biol.** 81, 193-214.

Guerette, B., Asselin, I., Vilquin, J.T. *et al.* **(1995)**. Lymphocyte infiltration follwing allo- and xenomyoblast transplantation in mdx mice. **Muscle Nerve** 18, 39-51.

Guerette, B., Skuk, D., Celestin, F. **(1997)**. Prevention by Anti-LFA-1 of acute myoblast death following transplantation. **J. Immunol.** 159, 2522-2531.

Gussoni, E., Pavlath, G.K. Lanctot, A.M. *et al.* **(1992)**. Normal dystrophin transcripts detected in Duchenne muscular dystrophy patients after myoblast transplantation. **Nature** 356, 435-438.

Hamamori, Y., Samal, B., Tian, J. *et al.* **(1994)**. Persistent erythropoiesis by myoblast transfer of erythropoietin cDNA. **Hum. Gene Ther.** 5, 1349-1356.

Heyck, H., Laudahn, G., Carsten, P.M. **(1996)**. Enzymaktivitatsbestimmungen bei Dystrophia musculorum progressiva. IV Mitteilung. **Klinisce Wochenschrift** 44, 695.

Hillman, A. **(1996)** Gene Therapy: Socioeconomic and Ethical Issues: A Roundtable Discussion. **Hum. Gene Ther.** 7, 1139-1144.

Hoeben, R.C. **(1995)**. Gene therapy for the haemophilias , current status. **The International Association of Biological Standardization.** 23, 27-29.

Hoffman, E.P., Brown, R.H., Kunkel, L.M. **(1987)**. Dystrophin , the protein product of the Duchenne muscular dystrophy locus. **Cell** 51, 919-928.

Hooper, C. **(1990)**. Duchenne therapy trials starting in U.S. Canada. **J. NIH Res.** 2, 30.

Huard, J., Bouchard, J.P. Roy, R. *et al.* **(1992)**. Human myoblast transplantation , preliminary results of 4 cases. **Muscle Nerve** 15, 550-560.

Huber, B. E., Lazo, J. S. (eds.) **(1994)**. **Gene Therapy for Neoplastic Diseases.** The New York Academy of Sciences. New York.

Jiao, S., Cheng, L., Wolff, J.A. *et al.* **(1993)**. Particle bombardment-mediated gene transfer and expression in rat brain tissue. **Bio/Technology** 11, 497-502.

Jones, P.H. **(1979)**. Implantation of cultured regenerate muscle cells into adult rat muscle. **Exp. Neurol.** 66, 602-610.

Kahan, B.D., Bach, J.F. eds. **(1988)**. Proceedings of the second international congress on cyclosporine, therapeutic use in transplantation. **Transpl. Proc.** 20, 1-1137.

Karlsson, S. **(1991)**. Treatment of genetic defects in hematopoietic cell function by gene transfer. **Blood** 78, 2481-2492.

Karpati, G., Ajdukovic, D. Arnold, D. *et. al.* **(1993)**. Myoblast transfer in Duchenne muscular dystrophy. **Ann. Neurol.** 34, 8-17.

Karpati, G., Pouliot, Y., Zubrzycka-Gaarn *et al.* **(1989)**. Dystrophin is expressed in mdx skeletal muscle fibers after normal myoblast implantation. **Am. J. Pathol.** 135, 27-32.

Katagiri, T. Yamaguchi, A., Komaki, M. *et al.* **(1994)**. Bone morphogenetic protien-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. **J. Cell Biol.** 127, 1755-1766.

Kessler, D.A., Siegel, J.P., Noguchi, P.D. *et al.* **(1993)**. Regulation of somatic cell-therapy and gene therapy by the Food and Drug Administration. **N. Engl. J. Med.** 329, 1169-1173.

Kinoshita, I., Vilquin, J.T., Guerette, B. *et al.* **(1994)**. Very efficient myoblast allotransplantation in mice under FK-506 immunosuppression. **Muscle Nerve** 17, 1407-1415.

Knowles, M.R., Hohneker, K., Zhou, Z.Q. *et al.* **(1995)**. A double blind vehicle-controlled study of adenoviral mediated gene transfer in the nasal epithelium of patients with cystic fibrosis. **N. Eng. J. Med.** 333, 823-831.

Koh, G.Y., Soonpaa, M.H., Klug, M.G. *et al.* **(1995)**. Stable fetal cardiomyocyte grafts in the hearts of dystrophic mice and dog. **J. Clin. Invest.** 96, 2034-2042.

Kornegay, J.N., Prattis, S.M., Bogan, D.J. *et al.* **(1992)**. Results of myoblast transplantation in a canine model of muscle injury. In , Kakulas, B.A., Howell, J.M.C., Roses, A.D., eds. **Duchenne muscular dystrophy. Animal models and genetic manipulation.** Raven Press. New York. pp. 203-212.

Langer, R, Vacanti, J.P. **(1993)**. Tissue engineering. **Science** 260, 920-926.

Lau, H.T., Yu, M., Fontana, A. *et al.* **(1996)**. Prevention of islet allograft rejection with engineered myoblast expressing FasL in mice. **Science** 273, 109-112.

Law, P. K. **(1978)**. Reduced regenerative capability of minced dystrophic mouse muscles. **Exp. Neurol.** 60, 231-243.

Law, P.K. **(1982)**. Beneficial effects of transplanting normal limb-bud mesenchyme into dystrophic mouse muscle. **Muscle Nerve** 5, 619-627.

Law, P.K. **(1994)**. **Myoblast Transfer: Gene Therapy for Muscular Dystrophy.** R.G. Landes Company, Austin, TX, p. 139-154.

Law, P.K. and Atwood, H.L. **(1972)**. Nonequivalence of surgical and natural denervation in dystrophic mouse muscle. **Exp. Neurol.** 34, 200-209.

Law, P.K., Atwood, H.L., McComas, A.J. **(1976)**. Functional denervation in the soleus muscle of dystrophic mice. **Exp. Neurol.** 51,434-443.

Law, P.K., Bertorini, T.E., Goodwin, T.G. *et al.* **(1990a)**. Dystrophin production induced by myoblast transfer therapy in Duchenne muscular dystrophy. **Lancet** 336, 114-115.

Law, P.K., Cosmos, E., Butler, J. *et. al.* **(1976)**. The absence of dystrophic characteristics in normal muscles successfully cross-reinnervated by nerves of dystrohpic genotype , physiological and cytochemical study of crossed solei of normal and dystrophic parabiotic mice. **Exp. Neurol.** 51, 1-21.

Law, P.K., Goodwin, T.G., Fang, Q. *et al.* **(1991a)**. Long-term improvement in muscle function, structure, and biochemistry following myoblast transfer in DMD. **Acta Cardiomiol.** 3, 281-301.

Law, P.K., Goodwin, T.G., Fang, Q. *et al.* **(1991b)**. Myoblast transfer therapy for Duchenne muscular dystrophy. **Acta Paediatr. Jpn.** 33, 206-215.

Law, P.K., Goodwin, T.G., Fang, Q. *et al.* **(1992)**. Feasibility, safety, and efficacy of myoblast transfer therapy on Duchenne muscular dystrophy boys. **Cell Transplantation** 1, 235-244.

Law, P.K., Goodwin, T.G., Fang, Q. *et al.* **(1993)**. Cell transplantation as an experimental treatment for Duchenne muscular dystrophy. **Cell Transplantation** 2, 485-505.

Law, P.K., Goodwin, T.G. Fang, Q. *et al.* **(1994a)**. Whole body myoblast transfer. **Transpl. Proc.** 26, 3381-3383.

Law, P.K., Goodwin, T.G., Fang, Q. *et al.* **(1995)**. Myoblast transfer , gene therapy for muscular dystrophy. **J. Cell Biochem.** p. 367.

Law, P.K., Goodwin, T.G., Fang, Q. *et al.* **(1996)**. Human gene therapy with myoblast transfer. **Mol. Biol. of the Cell.** 7, 3639.

Law, P.K., Goodwin, T.G., Fang, Q. *et al.* **(1997a)**. Human gene therapy with myoblast transfer. **Transpl. Proc.** 29, 2234-2237.

Law, P.K., Goodwin, T.G., Fang, Q., et al. **(1997b)**. First human myoblast transfer therapy continues to show dystrophin after 6 years. **Cell Transplantation** 6, 95-100.

Law, P.K., Goodwin, T.G., Fang, Q. *et al.* **(1997c)**. Myoblast transfer therapy (MTT) phase II clinical trials. **J. Physiol. Biochem.** 53, 80.

Law, P.K., Goodwin, T.G., Fang, Q. **(1997d)**. Advances in clinical trials of myoblast transfer therapy (MTT). **J. Neurol. Sci.** 150, S253.

Law, P.K., Goodwin, T.G., Li H.J. **(1988a)**. Histoincompatible myoblast injection improves muscle structure and function of dystrophic mice. **Transpl. Proc.** 20, 1114-1119.

Law, P.K., Goodwin, T.G., Li, H.J. *et al.* **(1990b)**. Myoblast transfer improves muscle genetics/structure; function and normalizes the behavior and life-span of dystrophic mice. In , Griggs, R.C., Karpati, G. eds. **Myoblast Transfer Therapy.** Plenum Press. New York. pp. 75-87.

Law, P.K., Goodwin, T.G., Li, H.J. *et al.* **(1990c)**. Plausible structural/ functional/behavioral/biochemical transformation following myoblast transfer therapy. In: Griggs, R.C., Karpati, G. eds. **Myoblast Transfer Therapy.** Plenum Press. New York. pp.241-250.

Law, P.K., Goodwin, T.G., Wang, M.G. **(1988b)**. Normal myoblast injections provide genetic treatment for murine dystrophy. **Muscle Nerve** 11, 525-533.

Law, P.K., Li, H., Chen, M. *et al.* **(1994b)**. Myoblast injection methods regulates cell distribution and fusion. **Tranplant. Proc.** 26, 3417-3418.

Law, P.K., Li, H.J., Goodwin, T.G. *et al.* **(1990d)**. Pathogenesis and treatment of hereditary muscular dystrophy. In , Kakulas, B.A., Mastaglia, F.L. eds.

Pathogenesis and Therapy of Duchenne and Becker Muscular Dystrophy. Raven Press. 101-118.

Law, P.K., Saito, A., Fleischer S. **(1983)**. Ultrastructural changes in muscle and motor end-plate of the dystrophic mouse. **Exp. Neurol.** 80, 361-382.

Law, P.K., Yap, J.L. **(1979)**. New muscle transplant method produces normal twitch tension in dystrophic muscle. **Muscle Nerve** 2, 356-363.

Leinwand, L.A. and Leiden, J.M. **(1991)**. Gene transfer into cardiac myocytes *in vivo*. **TCM.** 1, 271-276.

Li, R. K., Jia, Z.Q., Weisel, R.D. *et al.* **(1996)**. Cardiomyocyte transplantation improves heart function. **Ann Thorac. Surg.** 62, 654-661.

Massimino, M.L., Rapizzi, E., Cantini, M. et al. **(1997)**. ED2+ macrophages increase selectively myoblast proliferation in muscle cultures. **Biochem. Biophys. Res. Comm.** 235, 754-759.

Mendell, J.R., Kissel, J.T., Amato, A.A. *et. al.* **(1995)**. Myoblast transfer in the treatment of Duchenne muscular dystrophy. **N. Engl. J. Med.** 333, 832-838.

Miller, R.G., Pavlath, G., Sharma, K. *et al.* **(1992)**. Myoblast implantation in Duchenne muscular dystrophy , the San Francicso study. **Neurology** 42, 189-190.

Miyanohara, A., Johnson, P.A., Elam, R.L. *et al.* **(1992)**. Direct gene transfer to the liver with herpes simplex virus Type 1 vectors, transient production of physiologically relevant levels of circulating factor IX. **New Biol.** 4, 238-246.

Mokri, B., Engel, A.G. **(1975)**. Duchenne dystrophy , electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fiber. **Neurology** 25, 1111.

Morandi, L., Bernasconi, P., Gebbia, M. *et al.* **(1995)**. Lack of mRNA and dystrophin epxression in DMD patients three months after myoblast transfer. **Neuromusc. Disord.** 5, 291-295.

Morgan, J.E., Watt, D.J. Sloper, J.C. *et al.* **(1988)**. Partial correction of an inherited biochemical defect of skeletal muscle by grafts of normal muscle precursor cells. **J. Neurol. Sci.** 86, 137-147.

Morishita, R., Gibbons, G.H. Horiuchi, M. *et al.* **(1995)**. A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation *in vivo*. **Proc. Natl. Acad. Sci. USA** 92, 5855-5859.

Morsy, M.A., Caskey, C.T. **(1997)**. Safe gene vectors made simpler. **Nature Biotech.** 15, 17.

Murry, C.E., Wiseman, R.W., Schwartz, S.M. *et al.* **(1996)**. Skeletal myoblast transplantation for repair of myocardial necrosis. **J. Clin. Invest.** 98, 2512-2523.

Nerem, R.M., Sambanis, A. **(1995)**. Tissue engineering , from biology to biological substitutes. **Tissue Engineering** 1, 3-12.

Partridge, T.A., Grounds, M., Sloper, J.C. **(1978)**. Evidence of fusion between host and donor myoblasts in skeletal muscle grafts. **Nature** 273, 306-308.

Partridge, T.A., Morgan, J.E., Coulton, G.R. *et al.* **(1989)**. Conversion of mdx myofibers from dystrophin-negative to -positive by injection of normal myoblasts. **Nature** 337, 176-179.

Partridge, T.A. and Sloper, J.C. **(1977)**. A host contributios to the regeneration of muscle grafts. **J. Neurol. Sci.** 33, 425-435.

Puchalski, R.B., Fahl, W.E. **(1992)**. Gene transfer by electroporation, lipofection, DEAE-dextran transfection , compatibility with cell-sorting by flow cytometry. **Cytometry** 13, 23-30.

Ray, J., Gage, F.H. **(1992)**. Gene transfer in established and primary fibroblast cell lines , comparision of transfection methods and promoters. **Biotechniques** 13, 598-603.

Robinson, S.W., Cho, P.W., Levitsky, H.I. *et al.* **(1996)**. **Cell Transplantation** 5, 77-91.

Rosenfeld, M.A. and Collins, F.S. **(1996)**. Gene therapy for cystic fibrosis. **Chest** 109, 241-252.

Saito, A., Law, P.K., Fleischer, S. **(1983)**. Study of neurotrophism with ultrastructure of normal/dystrophic parabiotic mice. **Muscle Nerve** 6, 14-28.

Sautter, C., Waldner, H., Neuhaus-Url, G. *et al.* **(1991)**. Micro-targeting , high efficency gene transfer using a novel approach for the acceleration of micro-projectiles. **Bio/Technology** 9, 1080-1085.

Schwartz, E.R. **(1997)**. Tissue engineering focused ATP program. **Tissue Engineering** 3, 5-17.

Smith, T.A.G., Mehaffey, M.G. Kavda, D.B. *et al.* **(1993)**. Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice. **Nat. Genet.** 5, 397-402.

St. Louis, D., Verma, I.M. **(1988)**. An alternative approach to somatic cell gene therapy. **Proc. Natl. Acad. Sci. USA.** 85, 3150-3154.

Starzl, T.E., Thomson, A.W., Todo, S.N. *et al.* **(1991)**. Proceedings of the first international congress on FK 506. **Transpl. Proc.** 23, 2709-3380.

Stewart, M.J., Plautz, G.E., Del Buono, L. *et al.* **(1992)**. Gene transfer *in vivo* with DNA-liposome complexes , safety and acute toxicity in mice. **Hum. Gene Ther.** 3, 267-275.

Sunada, Y., Bernier, S.M., Utani, A. *et al.* **(1995)**. Identification of a novel mutant transcript of laminin a 2 chain gene responsible for muscular dystrophy and dysmyelination in dy^{2J} mice. **Hum. Mol. Genet.** 4, 1055-1061.

Teboul, L., Gaillard, D., Staccini, L. *et al.* **(1995)**.Thiazolidinediones and fatty acids convert myogenic cells into adipose-like cells. **J. Biol. Chem.** 270, 28183-28187.

Tremblay, J.P., Malouin, F., Roy, R. *et al.* **(1993)**. Results of a triple blind clinical study of myoblast transplantations without immunosuppressive treatment in young boys with Duchenne muscular dystrophy. **Cell Transplantation** 2, 99-112.

Trubetskoy, V.S., Torchilin, V.P., Kennel, S.J. *et al.* **(1992)**. Cationic liposomes enhance targeted delivery and expression of exogenous DNA mediated by N-terminal modified poly-L-lysine-antibody conjugate in mouse lung

endothelial cells. **Biochem. Biophy. Acta.** 1131, 311-313.

Van Meter, C.H., Claycomb, W.C., Delcarpio, J.B. *et al.* **(1995)**. Myoblast transplantation in the porcine model , a potential technique for myocardial repair. **J. Thorac. Cardiovasc. Surg.** 110, 1442-1448.

Vilquin, J.T., Kinoshita, I, Roy, R. *et al.* **(1995)**. Cyclosphosphamide immunosuppresion does not permit successful myoblast allotranplantation in mouse. **Neuromus. Disord.** 5, 511-517.

Watt, D.J. **(1982)**. Factors which affect the fusion of allogeneic muscle precursor cells *in vivo*. **Neuropath. Appl. Neurol.** 8, 135-147.

Watt, D.J., Lambert, K. Morgan, J.E. *et al.* **(1982)**. Incorporation of donor muscle precursor cells into an area of muscle regenration in the host mouse. **J. Neurol. Sci.** 57, 319-331.

Watt, D.J., Morgan, J.E., Partridge, T.A. **(1984)**. Long-term survival of allografted muscle precursor cells following a limited period of treatment with cyclosporin **A. Clin. Exp. Immunol.** 55, 419-426.

Wolff, J.A., Malone, R.W., Williams, P. *et al.* **(1990)**. Direct gene transfer into mouse muscle *in vivo*. **Science** 247, 1465-1468.

Wolff, J.A., Williams, P., Ascadi, G. *et al.* **(1991)**. Conditions affecting direct gene transfer into rodent muscle *in vivo*. **Biotechniques** 11, 474-485.

Yao, S.N., Smith, K.J., Kurachi, K. **(1994)**. Primary myoblast-mediated gene transfer , persistent expression of human factor IX in mice. **Gene Ther.** 1, 99-107.

EXHIBIT C

University of Pittsburgh

School of Medicine
Department of Molecular Genetics and Biochemistry
Biomedical Science Tower, Room E1240

Pittsburgh, Pennsylvania 15261
412-648-9570
Fax: 412-624-1401

Letter of Retraction and Apology which reads as follows:

On several occasions, I have made damaging statements regarding Dr. Peter K. Law and Cell Therapy Research Foundation of Memphis, Tennessee. These statements were made to members of the media and were carried over the University of Pittsburgh's internet site. Specifically, I made statements that impugned the personal character and scientific integrity of Dr. Law and Cell Therapy Research Foundation. I apologize publicly to Dr. Law and Cell Therapy Research Foundation for these statements and I hereby retract them. I wish Dr. Law and Cell Therapy Research Foundation the best of success in their efforts to find a treatment for muscular dystrophy with myoblast transfer therapy.

Eric P Hoffman, PhD

2/2/99
Date

EXHIBIT D

Myotubes Can Be Formed Within Implanted Adipose Tissue

A. Satoh, C. Labrecque, and J.P. Tremblay

THE TRANSPLANTATION of normal myoblasts to patients with hereditary muscle disease such as Duchenne muscular dystrophy (DMD) is a potentially promising therapeutic concept.[1–3] Injection of myoblasts from normal mouse[2,4] or human muscle[3] into growing or regenerating muscle of dystrophic (mdx) mice can lead to substantial expression of dystrophin, a cytoskeletal protein lacking in mdx mice and in patients with DMD.[5,6] One of the potential problems that may be encountered in the application of this approach to DMD is the progressive wasting of the DMD muscles. In later stages in DMD patients, a large part of the muscle mass has been replaced by connective and adipose tissues.[7] In this regard, although genetically and biochemically homologous to DMD,[6,8] the mdx mouse is an imperfect model for DMD. Like DMD patients, the mdx mouse suffers widespread degeneration of its skeletal muscle fibers[9]; however, the balance between degeneration of existing muscle fibers and regeneration of new muscle fibers results in an animal never severely weakened.[10] Although with increasing age mdx muscles do accumulate greater than normal amounts of collagen,[11] they do not commonly show the interfiber deposition of adipose and fibrous tissues so characteristic of older DMD patients. Following myoblast transplantation in these patients, the injected myoblasts have to form hybrid muscles or new muscles in and across these wasted muscles which are full of connective and adipose tissues. For this reason, it is important to clarify whether myoblasts can form myotubes and new muscle fibers in the fatty connective tissue. In this study, we inserted the fatty connective tissue into the hind limbs of mice and investigated whether myotubes and new muscle fibers can be formed in this transplanted fatty connective tissue. We demonstrated that myoblasts can invade the implanted fatty connective tissue and make myotubes and new muscle fibers across the entire implanted gap. The results imply that the myoblast transplantation procedure in the wasted muscles is capable of making myotubes or new muscle fibers even in such muscles.

MATERIALS AND METHODS

At 2 months of age, 16 male C57BL10J +/+ mice were subjected to fatty connective tissue implantation of the left hind limb tibialis anterior (TA) muscle. Fifty microliters of notexin (10 μg/mL) was first injected into the TA muscle to destroy muscle fibers and trigger regeneration. The following day, under ketamine-xylazine anesthesia, the central portion of the TA muscle was removed piece-by-piece from a small hole in the exterior side of TA muscle and fatty connective tissue derived from the axilla of the same mouse was inserted. The hole was carefully closed with suture and the skin incision was closed with sterile suture. After 7, 14, 21, and 28 days, four mice were perfused with saline under ketamine-xylazine anesthesia and the left TA muscles were isolated from the hind limb. Isolated muscles were immersed in sucrose (30% in PBS) for 12 hours. The specimens were frozen in liquid nitrogen. Eight-micrometer-thick cryostat sections were then prepared at the midpoint of the inserted adipose tissue.

RESULTS

Postimplantation Day 7

Muscle fibers were completely disrupted and implanted fatty connective tissue was observed at the center portion of the muscle (Fig 1a). A large number of cells were observed at the margin between stumps of the muscle fibers and implanted fatty connective tissue. Some of them made small myotubes (Fig 1b). The direction of some new myotubes appeared randomly at the margin. However, many myotubes in the inserted fatty connective tissue were directed from one side to the other side along the direction of the original muscle fibers.

Postimplantation Day 14

The myotubes observed in transplanted tissue increased in number and length, and some of them formed bundles.

These myotubes invaded deep into fatty connective tissue toward the other side. Almost all myotubes and bundles of myotubes were directed in the right direction in the implanted fatty connective tissue (Fig 1c).

Postimplantation Day 21

Many myotubes but few basophilic regenerating muscle fibers succeeded in extending across the entire implanted gap between the surviving stump of the muscles. The myotubes appeared more abundant than at 14 days. The diameter of the bundles of myotubes began to increase in size though the diameter of myotubes remained small (Fig 1d).

From the Laboratoire de Neurobiologie, Universite Laval, Hopital de l'Enfant-Jesus, Quebec, Canada.

Supported by grants from the Medical Research Council of Canada and the Muscular Dystrophy Association of Canada.

Address reprint requests to Jacques P. Tremblay, Centre de Recherche en Neurobiologie, Hopital de l'Enfant-Jesus, 1401, 18e Rue, Quebec (PQ) G1J 1Z4, Canada.

0041-1345/92/$3.00/+0

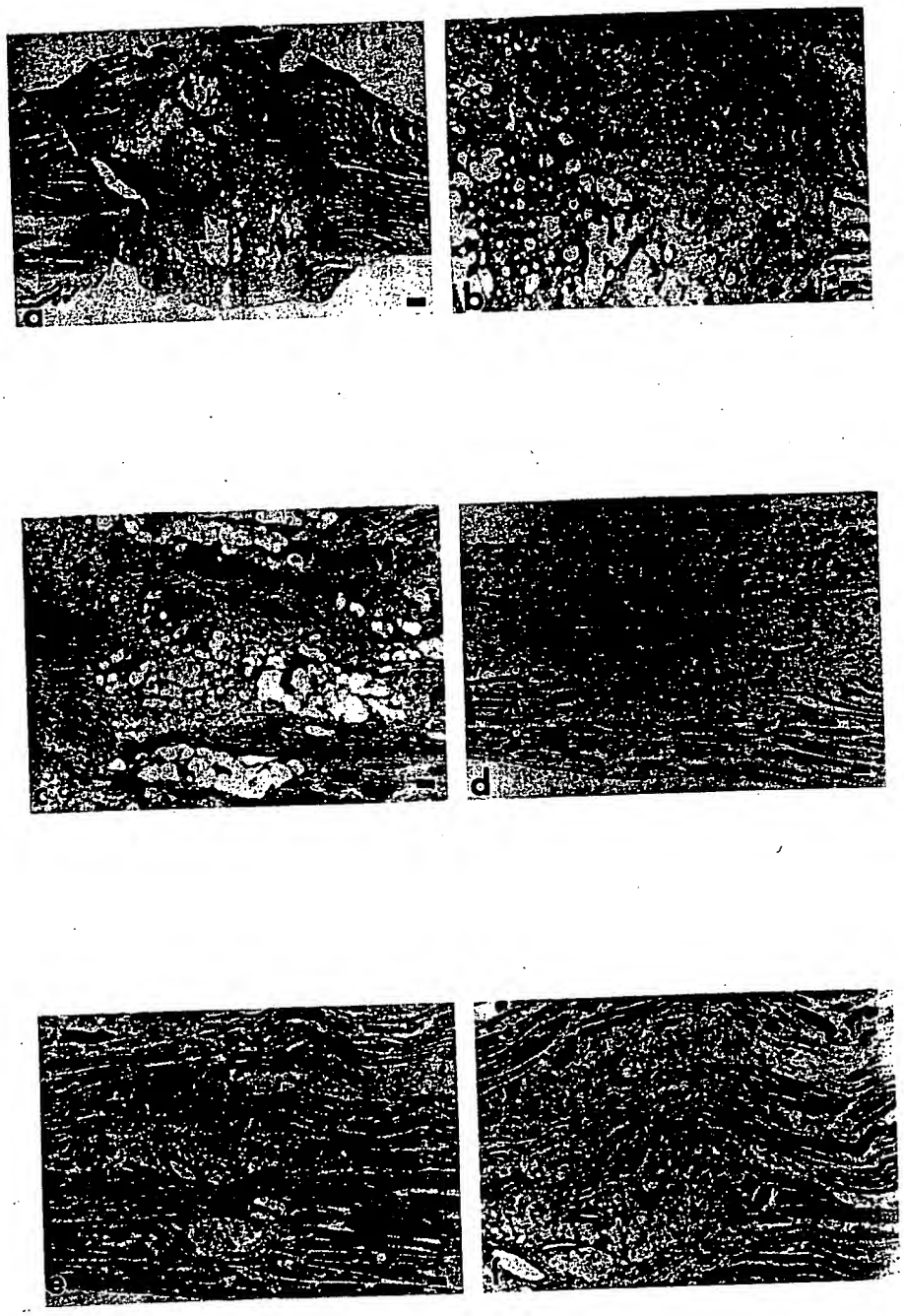


Fig 1. Longitudinal section 7 days after fatty connective tissue implantation. **(a)** Muscle fibers were completely disrupted and implanted fatty connective tissue was observed at the center portion of the muscle (bar = 200 μm). **(b)** A large number of cells were observed at the margin between the stump of muscle fibers and implanted fatty connective tissue. Some of these cells invaded into implanted tissue from both sides and some made small myotubes (bar = 50 μm). **(c)** Fourteen days after implantation. The myotubes observed in transplanted tissue increased in number and length, and invaded deep into fatty connective tissue toward the other side. Some myotubes formed bundles (bar = 50/μm). **(d)** Twenty-one days after implantation. Many myotubes and few regenerating muscle fibers succeeded in extending across the entire implanted gap between the surviving stumps of the muscles. The myotubes were increased in number and the diameter of the bundle of myotubes began to increase in size (bar = 100 μm). **(e and f)** Twenty-eight days after implantation. The myotubes and regenerated muscle fibers that extended across the implanted fatty connective tissue began to increase in number and the regenerated muscle fiber began to increase in diameter. The space between muscle stumps in which fatty connective tissue had been implanted became occupied by abundant myotubes and regenerating muscle fibers. These myoblasts and regenerated muscle fibers ran almost straight from one side to the other side across the implanted tissue [(e) bar = 100 μm, (f) bar = 50 μm].

Postimplantation Day 28

The myotubes and regenerated muscle fibers which extended across the implanted fatty connective tissue began to increase in number and the regenerated muscle fibers began to increase in diameter. The space between muscle stumps in which fatty connective tissue had been implanted became occupied by abundant myotubes and regenerating muscle fibers. These myotubes and regenerated muscle fibers ran almost straight from one side to the other side across the implanted tissue (Fig 1e and f).

DISCUSSION

In the later stages of DMD, a large part of muscle mass is replaced by the fatty connective tissue.[7] For the evaluation of the myoblast transplantation therapy in late stages of DMD, it is necessary to know whether myoblasts can

make new myotubes and new muscle fibers in these muscles. For this purpose, mdx mouse is not adequate. The model can be improved by locally high doses of x-irradiation which inhibits the regeneration of muscles. Thus, muscle fibers lose their closely packed arrangement and become progressively separated by loose connective tissue.[12] However, it takes a long time to make this model and the interfibrillar connective tissue is loose and not abundant enough to be a good model of wasted muscle in later stage of DMD. In this study, mature fatty connective tissue was inserted in the center portion of TA muscle. The TA muscle was therefore completely disrupted and the implanted fatty connective tissue occupied a large space at the center of the muscle (Fig 1a). This implanted tissue was mature and full of adipose and fibrous components and basal lamina was disrupted between the muscle stumps. For the recovery of the continuity of muscle, myoblasts from stumps of both sides have to invade the fatty connective tissue and form myotubes and new muscle fibers across the entire gap of that tissue. For this reason, this is one adequate model of the wasted DMD muscle which is full of fatty connective tissue. It has been demonstrated that muscles have great powers of regeneration in response to chemical and physical injury.[13–21] In this study, at 7 days after implantation, a large number of myoblasts already began to invade the implanted fatty connective tissue and some of them made myotubes (Fig 1b). At 21 days after implantation, many myotubes and some regenerated muscle fibers succeeded in extending across the entire implanted gap between disrupted muscles (Fig 1d). These results indicate that myoblasts have the potentiality to make myotubes across fatty connective tissue without basal lamina, and suggest that the myoblast transplantation procedure in the wasted DMD muscles has the possibility to make myotubes or new muscle fibers even in that wasted muscle.

ACKNOWLEDGMENTS

We thank Mr Francois Tardif for technical assistance.

REFERENCES

1. Mendel JL: Nature 339:584, 1989
2. Partridge TA, Morgan JE, Coulton GR, et al: Nature 337: 176, 1989
3. Karpati G, Pouliot Y, Zubrzycka-Gaarn E, et al: Am J Pathol 135:27, 1989
4. Morgan JE, Hoffman EP, Partrige TA: J Cell Biol 111:2437, 1990
5. Sugita H, Arahata K, Ishiguro T, et al: Proc Jpn Acad 64(ser B):37, 1988
6. Hoffman EP, Brown RH, Kunkel LM: Cell 51:919, 1987
7. Dubowitz V, Brook MH: Muscle Biopsy: A Modern Approach. Philadelphia, Saunders, 1973
8. Bulfield G, Siller WG, Weight PAL, et al: Proc Natl Acad Sci USA 81:1189, 1984
9. Carnwath JW, Shotton D: J Neurol Sci 80:39, 1987
10. Anderson JE, Ovalle WK, Bressler BH: Anat Rec 219:243, 1987
11. Marshall PA, Williams PE, Goldspink G: Muscle Nerve 12:528, 1989
12. Wakeford S, Watt DJ, Partridge TA: Muscle Nerve 14:42, 1991
13. Vracko R, Benditt E: J Cell Biol 55:406, 1972
14. Hansen-Smith FM, Carlson BM: J Neurol Sci 41:149, 1979
15. Allbrook DB: Muscle Nerve 4:234, 1981
16. Alameddine HS, Hantai D, Dehaupas M, et al: Neuromusc Dis 1:143, 1991
17. Jarvinen M, Sorvari T: Acta Pathol Microbiol Scand Sect A83:259, 1975
18. Jarvinen M: Acta Pathol Microbiol Scand Sect A83:269, 1975
19. Grounds MD, McGeachie JK: Muscle Nerve 1:305, 1990
20. McGeachie JK, Grounds MD: Cell Tissue Res 248:125, 1987
21. Hurme T, Lehto M, Falck B, et al: Acta Physiol Scand 142:443, 1991

TRANSPLANTATION PROCEEDINGS

Proceedings of the

TENTH ANNIVERSARY OF THE COLLABORATIVE TRANSPLANT STUDY AND 100,000 CTS TRANSPLANTS

May 10–13, 1992
Heidelberg, Germany

Guest Editor
GERHARD OPELZ
Ruprecht-Karls Universität Heidelberg
Heidelberg, Germany

ALSO IN THIS ISSUE

First International Congress of the
CELL TRANSPLANT SOCIETY
May 31—June 3, 1992
Pittsburgh, Pennsylvania

Guest Editors
CAMILLO RICORDI
ANGUS W. THOMSON
SUZANNE T. ILSTAD
PATRICIA B. CARROLL
THOMAS E. STARZL
Pittsburgh, Pennsylvania

APPLETON & LANGE
25 Van Zant Street
P.O. Box 5630
Norwalk, CT 06856
Simon & Schuster Business and Professional Group
ISSN 0041-1345 3TPFA

EXHIBIT E

ELSEVIER

Cell Transplantation, Vol. 6, No. 1, pp. 95–100, 1997

0963-6897/97 $17.00 + .00

PII S0963-6897(96)00138-8

Commentary

FIRST HUMAN MYOBLAST TRANSFER THERAPY CONTINUES TO SHOW DYSTROPHIN AFTER 6 YEARS

PETER K. LAW,[1] TENA G. GOODWIN, QIUWEN FANG, TERRY L. HALL, TOM QUINLEY, GEORGE VASTAGH, VIJAYA DUGGIRALA, CHARLES LARKIN, JERRY ANN FLORENDO, LAWRENCE LI, TUNJA JACKSON, T. J. YOO, NANCY CHASE, MICHAEL NEEL, TIM KRAHN, AND RANDALL HOLCOMB

Cell Therapy Research Foundation, Memphis, TN, USA

☐ **Keywords — Myoblast transfer; Dystrophin; Gene Therapy; Duchenne muscular dystrophy.**

Gene therapy encompasses interventions that involve deliberate alteration of the genetic material of living cells to diagnose, prevent, or treat disease (28). The administration of cells that have undergone ex vivo genetic manipulation is considered a combination of somatic cell therapy and gene therapy (15). Although the majority of human gene therapy trials to date have used this combination approach, gene therapy products have also been administered directly to subjects to modify cells in vivo (28). One of these latter gene therapy products is myoblasts, and because myoblasts are cells, myoblast transfer therapy (MTT, U.S. Patent # 5,130,141) is a combination of somatic cell therapy and gene therapy designed to effect gene transfer in vivo.

The use of myoblasts as gene transfer vehicles dates as early as 1978 (31,32,42). In mammals, myoblasts are the only cells that divide extensively, migrate, fuse naturally to form syncytia, lose MHC-1 antigens soon after fusion, and develop to occupy 50% of the body weight in humans. These combined properties rendered myoblasts ideal for gene transfer. Expression of a foreign gene requires appropriate integration and regulation involving numerous cofactors, many of which are transient during embryonic development (33). Natural transduction of the normal genome following MTT will ensure replacement of dystrophin and related proteins involving polygenomic interaction in Duchenne muscular dystrophy (DMD).

The first published conceptual approach, dated 1978, to incorporate the "missing" gene and its product(s) into genetically defective cells of adult mammals utilized cell transplantation and natural cell fusion to strengthen hereditary degenerative muscles (31). By 1979, normal myogenic cell transfer was shown to have improved the function of single muscles of the dystrophic mice to normal (42). MTT has since been shown to have improved the cell genetics, structure, muscle function, animal behavior, and life span of the $dy^{2J}dy^{2J}$ dystrophic mice (32,40,41). With the discovery in 1987 of dystrophin, the absence of which characterizes DMD and mdx mouse dystrophy, a new biochemical marker became available to demonstrate MTT efficacy in DMD and mdx mice (8,47). These pioneering animal experiments led to clinical trials on DMD boys.

The first MTT on a DMD boy occurred on February 15, 1990. It was the first human gene therapy clinical trial (23). Cultured myoblasts capable of natural cell fusion were used as vehicles to deliver their complete normal genomes into DMD myofibers to repair genetic defects. As a cell therapy, MTT was to replenish the degenerated myofibers with normal ones developed through fusion among donor myoblasts (40,41). In addition to fulfilling their primary muscle-building mission, the myoblasts served as the source and the transfer vehicles of normal genes to correct the gene defects of DMD.

The study protocol received approval from 1) the Institutional Review Board of the University of Tennessee Memphis (UTM); 2) the Scientific Advisory and Administrative Committees of the Clinical Research Center in

ACCEPTED 5/9/96.
[1]Correspondence should be addressed to P. Law, Ph.D., Cell Therapy Research Foundation, 1770 Moriah Woods Blvd., Suite 18, Memphis, TN 38117.

UTM; 3) the LeBonheur Children's Medical Center Institutional Review Board; and 4) the UTM Data and Safety Monitoring Board.

The safety and efficacy of MTT was assessed by injecting the left extensor digitorum brevis (EDB) muscle of a 9-yr-old adopted boy having DMD with about 8×10^6 myoblasts. Donor myoblasts were cloned from satellite cells derived from a 1 g rectus femoris biopsy specimen of the normal, adoptive father. The only immunosuppressive agent administered was cyclosporine at a dose of 5–7 mg/kg body weight divided into two daily oral doses. The dosage was varied to maintain serum trough concentrations in the range of 100–150 ng/mL for 90 days.

Donor myoblasts survived, developed, and produced dystrophin in myofibers biopsied from the myoblast-injected EDB 92 days later. Dystrophin was not found in the contralateral muscle sham-injected with an equal volume (0.4 mL) of the carrier solution. This first case suggests that MTT offered a safe and effective means for alleviating biochemical deficit(s) inherent in muscles of DMD (34).

An often-asked question is how long can the myoblasts survive if they succeed in engrafting. To this end we have rebiopsied the myoblast- and sham-injected EDB muscles of the referenced world's first subject 6 yr after receiving MTT. Throughout this period these muscles did not receive any additional myoblasts or any other treatment.

Figure 1A and C shows the absence of immunocytochemical dystrophin in the control muscle sham-injected with 0.4 mL of the carrier saline. Dystrophin was present in the contralateral, myoblast-injected muscle (Fig. 1B and D). It was immunocytochemically localized at the sarcolemma using the method of Bonilla et al. (6). Although over 95% of the biopsied myofibers exhibited dystrophin, they are irregular in shape and size. Many appeared oval and small when compared to the polygonal, regularly sized normal control myofibers that are closely packed with little intercellular connective tissues (Fig. 1E and F).

At no time during the 6 yr after myoblast injection was there any sign of erythema, swelling, tenderness, or inflammation at the injection sites. Subsequent to this initial MTT in 1990, the subject received an additional 5×10^9 myoblasts in 22 major muscles in both legs in 1991, and another 25×10^9 myoblasts in 64 major muscle groups in both upper and lower bodies in 1994. In neither case were myoblasts administered close to the feet where the EDB muscles are located.

The result indicates that donor myoblasts survived, developed, and produced dystrophin within 92 days after MTT (34), and that the dystrophin-positive myofibers survived almost 6 yr. A reasonable assumption is that dystrophin prevented these fibers from undergoing degeneration, but such contention still awaits more definitive evidence. Despite the presence of dystrophin, the small size and oval shape of some of these myofibers suggest that they might not function normally, possibly due to atrophy as a result of reduced muscle activities. Nonetheless, the production of the structural protein dystrophin 6 yr after MTT provides confirmatory evidence of the correction of the primary gene defect of DMD. MTT appears to be a safe and effective gene therapy capable of producing long-term effect. Six years is the longest period for which any gene therapy has been followed with positive result.

One possible explanation of the presence of dystrophin in the myoblast-injected muscle is that reverse mutation might have occurred early during the development of this dystrophic muscle, thereby producing a high proportion of dystrophin-positive fibers. However, reverse mutation causing dystrophin production in adjacent myofibers occupying over 95% of the muscle biopsy with over 120 myofibers as shown in Fig. 1B has not been reported. Reverse mutation as published in the literature has accounted for no more than six dystrophin-positive fibers in similar sections.

To reconcile the current positive result with the less convincing ones reported by others (20,24,26,43–45,51) several issues need to be addressed.

To begin with, the use of large quantities of pure live myoblasts is a prerequisite of successful MTT. Besides Law's study (37), there is no published *pictorial* evidence to substantiate the purity, myogenicity, and viability of the injected myoblasts as claimed.

Myoblast cultures are usually contaminated with fibroblast overgrowth. MTT with such impure culture could lead to deposition of connective tissues rather than myofiber production. Culturing 25 billion pure human myoblasts for MTT from 2 g of muscle biopsy has only been reported by Law et al. (39). Other teams work at ranges of hundreds of millions of myoblasts.

In most studies (20,26,43–45) myoblasts were transported frozen, chilled, or at room temperature for about 2 h from the site of harvest before being injected. Because myoblasts have a high metabolic rate, they could not have survived for 2 h without significant nutrients, oxygen, and proper pH, being closely packed in saline within a vial for transport. Our myoblasts were injected into the subject within 30 min of harvest, at the same location without transport.

MTT studies that reported failure (20,26,43–45) subscribed to the fallacy of making 55 to 330 injections into a muscle the size of an egg, traumatizing indiscriminately the underlying nerves, muscle, and vasculature. These injection traumas boosted macrophage access and host immune responses (19). They also induced fibrosis

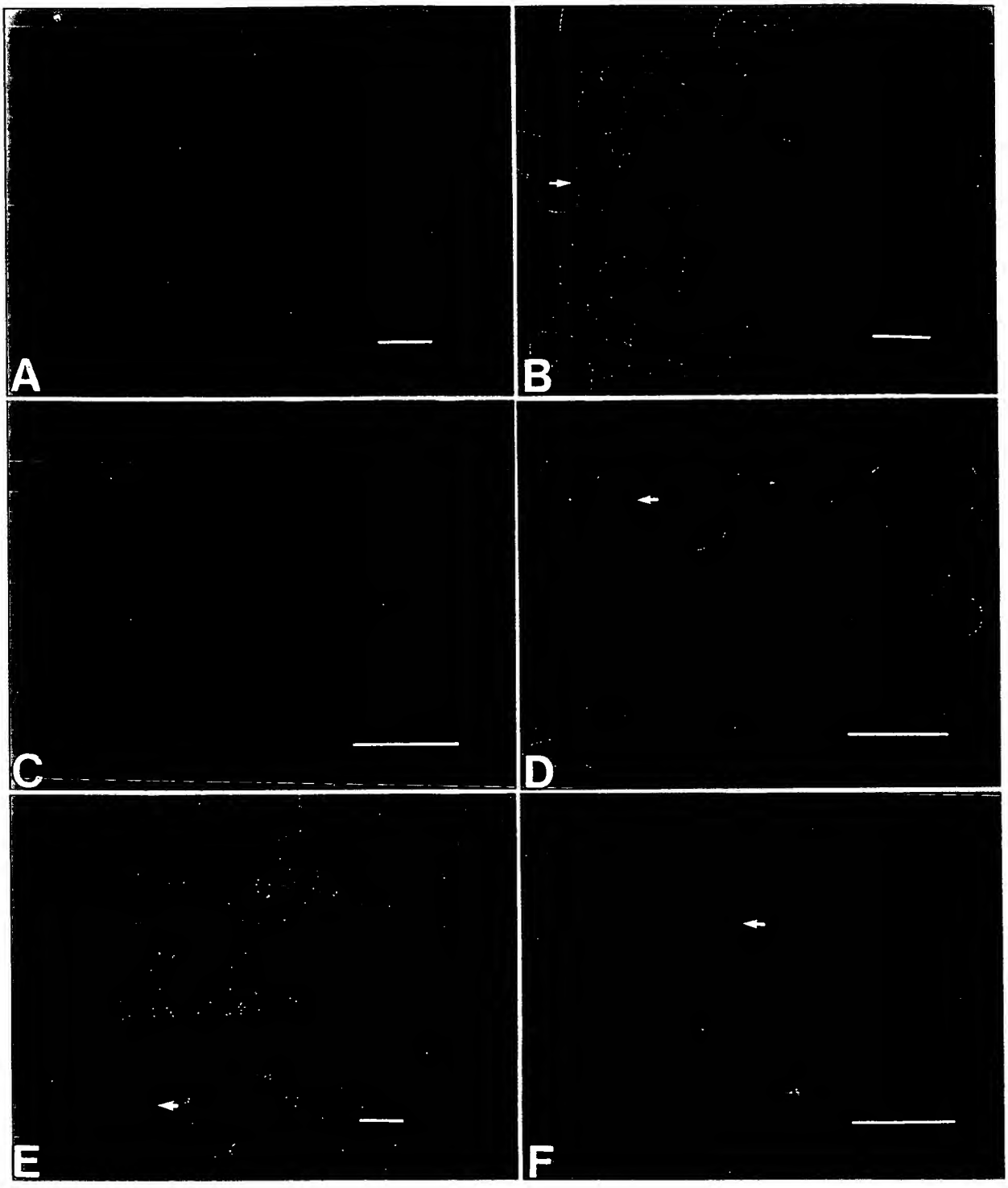


Fig. 1. Immunocytochemical demonstration of dystrophin in DMD muscles 6 yr after MTT. Dystrophin absent in sham-injected EDB muscle (A,C), but present in the contralateral myoblast-injected muscle (B,D). Dystrophin was immunocytochemically localized at the sarcolemma (arrows). Dystrophin demonstrated at low (E) and high (F) magnification in normal control muscle. Cross-sections; bar = 100 μm.

(9). Surviving myoblasts fused within 3 wk in small mouse muscles (8). A nerve with multiple trauma could not regenerate soon enough through scar and connective tissues to innervate the newly formed myotubes in a large human dystrophic muscle. Stabilization of muscle contractile properties in a similar situation is achieved by 60 days in the rat, and functional return is incomplete (7). Noninnervated myotubes died within 1 wk. Whatever few myotubes that developed in the unsuccessful MTT studies (20,26,43–45) could not compensate for the traumatized myofibers.

With eight injections we delivered 5 to 8 × 10^8 pure myoblasts into the biceps brachii without nerve injury (39). Contrarily, in Mendell's study, 55 sites, each 5 mm apart, distributed in 11 rows and 5 columns, were injected throughout the depth of each biceps of 5- to 9-yr-old boys (43). This was repeated monthly for 6 mo. Axonal sprouts, myotubes, and neuromuscular junctions that take 6 wk to mature (17) were repeatedly traumatized by a total of 330 injections until the biceps, with or without myoblast/cyclosporine, were irreversibly damaged or destroyed. The result—no functional difference between myoblast- and sham-injected muscles (43). How myoblasts escaped rejection and produced donor-derived dystrophin in two nonimmunosuppressed patients (#6 and #12) in that study cannot be explained. One would predict immune rejections and failure considering monthly repetition of large numbers of injections of histoincompatible myoblasts without cyclosporine.

Once injected, the myoblasts are subjected to scavenger hunt by macrophages for up to 3 wk. This is because myoblasts exhibit major histocompatibility complex class I (MHC-1) surface antigens (16,18) that eventually become absent after cell fusion (12). The latter occurs between 1 to 3 wk after myoblast injection (8). An allowance in the number of injected myoblasts has to be made to satisfy the unavoidable scavenger process. As reflected in the small numbers of myoblasts injected in unsuccessful studies, it appears that either such allowance was not considered or that the teams were not able to produce larger quantities of pure myoblasts.

The less successful MTT teams focused on immunosuppression to prevent T-lymphocyte proliferation and antibody production without overcoming the primary hurdle of providing enough pure and live myoblasts. A basic study indicates that cyclophosphamide did not permit myoblast engraftment in mouse (52). Without this prior knowledge, a MTT clinical trial was conducted without success using cyclophosphamide immunosuppression (26). Cyclosporine (34) and potentially FK506 (29) remain the immunosuppressants of choice for MTT. Results could have been more positive if either was employed in the study of Tremblay et al. (24,51).

All gene therapies involving viral vectors are still in Phase I clinical trials to determine safety. The gene therapy protocol for correction of adenosine deaminase (ADA) deficiency began on September 14, 1990 (2,3,11), 2 mo after MTT correction of DMD gene defect was published (34). In the ADA protocol, T cells from a patient with a severe combined immunodeficiency disorder (SCID) were transduced with functional ADA genes ex vivo and returned to the patient after expansion through culture. In the MTT protocol, primary culture of myoblasts derived from a muscle biopsy of a normal donor was injected into a muscle of the DMD subject to produce in vivo nuclear complementation. Both gene therapies utilize cell transplantation to treat diseases.

Today, the five subjects who underwent two ADA protocols with multiple readministration continue to be on enzyme replacement therapy, polyethylene glycol-conjugated ADA, and it is questionable as to whether the ADA gene therapy is effective. Over 130 DMD boys have received MTT with no adverse reactions since February 15, 1990. Functional, histologic, and biochemical improvements of DMD muscles have been reported (20,24,34–38,51). MTT on DMD is the most advanced form of gene therapy in which the myoblasts that are endogenous to the human body are used to effect gene transfer. We are conducting the first and only FDA-permitted Phase II MTT clinical trial on DMD under an investigational new drug application (IND), injecting 64 muscles of the upper and lower bodies with 25 billion myoblasts.

The foremost correction of primary gene defect in human (34), published on July 14, 1990, fueled immense enthusiasm of using normal and transduced myoblasts as gene delivery vehicles (5,31,32,40–42). In addition to clinical trials on Duchenne muscular dystrophy (16,20,24,26,35–38,43–45), MTT has found potential application in cardiomyopathy (10,30,49), restenosis (46), diabetes mellitus (48), human growth hormone deficiency (4,14), hemophilias (13,53), anemia (21), bone degeneration (27), Parkinsonism (25), soft tissue augmentation (50), and muscle trauma (1). Undoubtedly MTT has produced a new frontier in medicine with promise of treating hereditary and acquired diseases.

Acknowledgments — This work was supported by public donations. We gratefully acknowledge Susan Kenny for typing the manuscript.

REFERENCES

1. Alameddine, H.S.; Louboutin, J.P.; Dehaupas, M.; et al. Functional recovery induced by satellite cell grafts in irreversibly injured muscles. Cell Transplant. 3:3–14; 1994.

2. Anderson, W.F. The ADA human gene therapy clinical protocol. Hum. Gene Ther. 1:327–362; 1990.
3. Anderson, W.F. Human gene therapy. Science 256:808–813; 1992.
4. Barr, E.; Leiden, J.M. Systemic delivery of recombinant proteins by genetically modified myoblasts. Science 254: 1507–1509; 1991.
5. Blau, H.M.; Springer, M.L. Muscle-mediated gene therapy. N. Engl. J. Med. 333:1554–1556; 1995.
6. Bonilla, E.; Samitt, C.E.; Miranda, A.F.; et al. Duchenne muscular dystrophy: Deficiency of dystrophin at the muscle cell surface. Cell 54:447–452; 1988.
7. Carlson, B.M. The regeneration and transplantation of skeletal muscle. In: Seil, F., ed. Nerve, organ, and tissue regeneration: Research perspectives. New York: Academic Press; 1983:431–454.
8. Chen, M.; Li, H.J.; Fang, Q.; et al. Dystrophin cytochemistry in mdx mouse muscles injected with labeled normal myoblasts. Cell Transplant. 1:17–22; 1992.
9. Chen, S.S.; Chien, C.H.; Yu, H.S. Syndrome of deltoid and/or gluteal fibrotic contracture; An injection myopathy. Acta Neurol. Scand. 78:167–176; 1988.
10. Chiu, R.C.-J.; Zibaitis, A.; Kao, R.L. Cellular cardiomyoplasty: Myocardial regeneration with satellite cell implantation. Ann. Thorac. Surg. 60:12–18; 1995.
11. Culver, K.W.; Osborne, W.R.; Miller, A.D.; et al. Correction of ADA deficiency in human T lymphocytes using retroviral-mediated gene transfer. Transplant. Proc. 23: 170–171; 1991.
12. Daar, A.S.; Fuggle, S.V.; Fabre, J.W.; et al. The detailed distribution of HLA-A,B,C antigens in normal human organs. Transplantation 38:287–298; 1984.
13. Dai, Y.; Roman, M.; Naviaux, R.K.; et al. Gene therapy via primary myoblasts: Long-term expression of factor IX protein following transplantation in vivo. Proc. Natl. Acad. Sci. USA 89:10892–10895; 1992.
14. Dhawan, J.; Pan, L.C.; Pavlath, G.K.; et al. Systemic delivery of human growth hormone by injection of genetically engineered myoblasts. Science 254:1509–1512; 1991.
15. Epstein, S.L. Regulatory concerns in human gene therapy. Hum. Gene Ther. 2:243–249; 1991.
16. Fang, Q.; Chen, M.; Li, H.J.; et al. MHC-1 antigens on cultured human myoblasts. Transplant. Proc. 26:3467; 1994.
17. Fex, S.; Jirmanova, I. Innervation by nerve implants of "fast" and "slow" skeletal muscles of the rat. Acta Physiol. Scand. 76:257–269; 1969.
18. Friedlander, M.; Fischman, D.A. Immunological studies of the embryonic muscle cell surface. Antiserum to the prefusion myoblast. J. Cell Biol. 81:193–214; 1979.
19. Guerette, B.; Asselin, I.; Vilquin, J.T.; et al. Lymphocyte infiltration following allo- and xenomyoblast transplantation in mdx mice. Muscle Nerve. 18:39–51; 1995.
20. Gussoni, E.; Pavlath, G.K.; Lanctot, A.M.; et al. Normal dystrophin transcripts detected in Duchenne muscular dystrophy patients after myoblast transplantation. Nature 356: 435–438; 1992.
21. Hamamori, Y.; Samal, B.; Tian, J.; et al. Persistent erythropoiesis by myoblast transfer of erythropoietin cDNA. Hum. Gene Ther. 5:1349–1356; 1994.
22. Hamamori, Y.; Samal, B.; Tian, J.; et al. Myoblast transfer of human erythropoietin gene in a mouse model of renal failure. J. Clin Invest. 95:1808–1813; 1995.
23. Hooper, C. Duchenne therapy trials starting in U.S., Canada. J. NIH Res. 2:30; 1990.
24. Huard, J.; Bouchard, J.P.; Roy, R.; et al. Human myoblast transplantation: Preliminary results of 4 cases. Muscle Nerve 15:550–560; 1992.
25. Jiao, S.; Schultz, E.; Wolff, J.A. Intracerebral transplants of primary muscle cells: A potential platform for transgene expression in the brain. Brain Res. 575:143–147; 1992.
26. Karpati, G.; Ajdukovic, D.; Arnold, D.; et al. Myoblast transfer in Duchenne muscular dystrophy. Ann. Neurol. 34:8–17; 1993.
27. Katagiri, T.; Yamaguchi, A.; Komaki, M.; et al. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J. Cell Biol. 127:1755–1766; 1994.
28. Kessler, D.A.; Siegel, J.P.; Noguchi, P.D.; et al. Regulation of somatic cell-therapy and gene therapy by the Food and Drug Administration. N. Engl. J. Med. 329:1169–1173; 1993.
29. Kinoshita, I., Vilquin, J.-T.; Guerette, B.; et al. Very efficient myoblast allotransplantation in mice under FK-506 immunosuppression. Muscle Nerve 17:1407–1415; 1994.
30. Koh, G.Y.; Soonpaa, M.H.; Klug, M.G.; et al. Stable fetal cardiomyocyte grafts in the hearts of dystrophic mice and dogs. J. Clin. Invest. 96:2034–2042; 1995.
31. Law, P.K. Reduced regenerative capability of minced dystrophic mouse muscles. Exp. Neurol. 60:231–243; 1978.
32. Law, P.K. Beneficial effects of transplanting normal limb-bud mesenchyme into dystrophic mouse muscles. Muscle Nerve 5:619–627; 1982.
33. Law, P.K. Misrepresentation conspires against potential treatment for muscular dystrophy. IRB 17:4–6; 1995.
34. Law, P.K.; Bertorini, T.E.; Goodwin, T.G.; et al. Dystrophin production induced by myoblast transfer therapy in Duchenne muscular dystrophy. Lancet 336:114–115; 1990.
35. Law, P.K.; Goodwin, T.G.; Fang, Q.; et al. Long-term improvement in muscle function, structure and biochemistry following myoblast transfer in DMD. Acta Cardiomiol. 3:281–301; 1991.
36. Law, P.K; Goodwin, T.G.; Fang, Q.; et al. Myoblast transfer therapy for Duchenne muscular dystrophy. Acta Paediatr. Jpn. 33:206–215; 1991.
37. Law, P.K.; Goodwin, T.G.; Fang, Q.; et al. Feasibility, safety, and efficacy of myoblast transfer therapy on Duchenne muscular dystrophy boys. Cell Transplant. 1:235–244; 1992.
38. Law, P.K.; Goodwin, T.G.; Fang, Q.; et al. Cell transplantation as an experimental treatment for Duchenne muscular dystrophy. Cell Transplant. 2:485–505; 1993.
39. Law, P.; Goodwin, T.; Fang, Q.; et al. Whole body myoblast transfer. Transplant. Proc. 26:3381–3383; 1994.

40. Law, P.K.; Goodwin, T.G.; Li, H.J. Histoincompatible myoblast injection improves muscle structure and function of dystrophic mice. Transplant. Proc. 20:1114–1119; 1988.
41. Law, P.K.; Goodwin, T.G.; Wang, M.G. Normal myoblast injections provide genetic treatment for murine dystrophy. Muscle Nerve 11:525–533, 1988.
42. Law, P.K.; Yap, J.L. New muscle transplant method produces normal twitch tension in dystrophic muscle. Muscle Nerve 2:356–363; 1979.
43. Mendell J.R.; Kissel, J.T.; Amato, A.A.; et al. Myoblast transfer in the treatment of Duchenne's muscular dystrophy. N. Engl. J. Med. 333:832–838; 1995.
44. Miller, R.G.; Pavlath, G.; Sharma, K.; et al. Myoblast implantation in Duchenne muscular dystrophy: The San Francisco study. Neurology 42:189–190; 1992.
45. Morandi, L.; Bernasconi, P.; Gebbia, M.; et al. Lack of mRNA and dystrophin expression in DMD patients three months after myoblast transfer. Neuromusc. Disord. 5: 291–295; 1995.
46. Morishita, R.; Gibbons, G.H.; Horiuchi, M.; et al. A gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation in vivo. Proc. Natl. Acad. Sci. USA 92:5855–5859, 1995.
47. Partridge, T.A.; Morgan, J.E.; Coulton, G.R.; et al. Conversion of mdx myofibers from dystrophin-negative to -positive by injection of normal myoblasts. Nature 337: 176–179; 1989.
48. Simonson, G.D.; Groskreutz, D.J.; Gorman, C.M.; et al. Synthesis and processing of genetically modified human proinsulin by rat myoblast primary cultures. Hum. Gene Ther. 7:71–78; 1996.
49. Soonpaa, M.H.; Koh, G.Y.; Klug, M.G.; et al. Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. Science 264:98–101; 1994.
50. Teboul, L.; Gaillard, D.; Staccini, L.; et al. Thiazolidinediones and fatty acids convert myogenic cells into adipose-like cells. J. Biol. Chem. 270:28183–28187; 1995.
51. Tremblay, J.P.; Malouin, F.; Roy, R.; et al. Results of a triple blind clinical study of myoblast transplantations without immunosuppressive treatment in young boys with Duchenne muscular dystrophy. Cell Transplant. 2:99–112; 1993.
52. Vilquin, J.T.; Kinoshita, I.; Roy, R.; et al. Cyclosphosphamide immunosuppression does not permit successful myoblast allotransplantation in mouse. Neuromusc. Disord. 5: 511–517; 1995.
53. Yao, S.N.; Smith, K.J.; Kurachi, K. Primary myoblast-mediated gene transfer: persistent expression of human factor IX in mice. Gene Ther. 1:99–107; 1994.

EXHIBIT F

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
1401 Rockville Pike
Rockville MD 20852-1448

OCT 16 1998

Our Reference: BB-IND 5108

Cell Therapy Research Foundation
Attention: Peter K. Law, Ph.D., Chairman
1770 Moriah Woods Boulevard, Suite 18
Memphis, TN 38117

Dear Dr. Law:

Reference is made to your **Investigational New Drug Application (IND)** for "Cultured Allogeneic Myoblasts. and Cyclosporin (Sandoz)." We also refer to your submission of August 18, 1998, received on August 19, 1998, requesting designation as a Fast Track Product pursuant to Section 506 of the Food, Drug, and Cosmetic Act (the Act).

We have reviewed your request and we are designating as a Fast Track development program the investigation of cultured allogeneic myoblasts for delay or prevention of severe disability and/or death in patients with Duchenne Muscular Dystrophy.

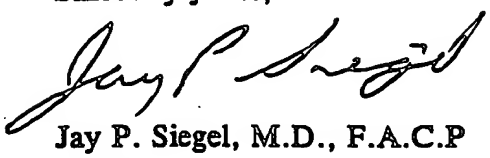
Please note that if the clinical development program you pursue does not continue to meet the criteria for Fast Track designation, the application will not be reviewed under the Fast Track program.

Under the FDA Modernization Act of 1997, designation as a Fast Track product for a new drug or biological product means that FDA will take such actions as are appropriate to expedite the development and review of the application for approval of such product. FDA may also evaluate for filing and commence review of portions of an application for approval of a Fast Track product under certain conditions.

FDA is the process of preparing detailed guidance on the provisions of Section 112 of the FDA Modernization Act of 1997 as required by the Act. Until such time as the guidance is publicly available, please contact our office in order to receive guidance on the development and review of your product and how these provisions will be applied. We look forward to working with you to expedite the development and review of this promising proposed use of the product.

If you any have questions, please contact Jeanne Delasko, Division of Application Review and Policy, at (301) 827-5101.

Sincerely yours,



Jay P. Siegel, M.D., F.A.C.P
Director
Office of Therapeutics
Research and Review
Center for Biologics
Evaluation and Research